NOVEL *DKR* POLYPEPTIDES

This application is a divisional of U.S.S.N. 09/161,241, filed September 25, 1998, which is hereby incorporated by reference.

Field of the Invention

This invention relates generally to novel genes encoding proteins that have use as anti-cancer therapeutics.

BACKGROUND

Related Art

One of the hallmarks of cells that have become cancerous is the change in the gene expression pattern in those cells as compared to normal, non-cancerous cells. An intricate series of cell signaling events leads to this so called "differential gene expression", resulting in conversion of a normal cell to a cancer cell (also known as "oncogenesis" or "cell transformation"). A number of cell signaling pathways have been implicated in the process of cell transformation, such as, for example, the cadherin pathway, the *delta/jagged* pathway, the *hedgehog/sonic hedgehog* pathway, , and the *wnt/wingless* pathway (Hunter, *Cell*, 88:333-346 [1997]; Currie, *J. Mol. Med.*, 76:421-433 [1998]; Peifer, *Science*, 275:1752-1753 [1997]. Interestingly, these same pathways are involved in cell morphogenesis, or cell differentiation, during embryo development (Hunter, supra; Cadigan *et al.*, *Genes and Develop.*, 11:3286-3305 [1997]).

The *wnt* genes encode glycoproteins that are secreted from the cell. These glycoproteins are found in both vertebrate and invertebrate organisms. Currently, there are at least 20 *wnt* family members, and these members are believed to function variously in control of growth and in tissue differention. Recently, discovery of a novel gene was identified in *Xenopus* and mouse and has been termed *dickkopf-1* (*"dkk-1"*). This gene is purportedly a potent antagonist of *wnt-8* signaling (Glinka *et al., Nature*, 391:357-362 [1998]). Interestingly, this gene is also purportedly involved in morphogenesis in the developing embryo (Glinka *et al., supra*). This gene thus represents a novel growth factor which may be useful in tissue regeneration, and also represents a means for potentially inhibiting cell transformation via *wnt* signaling.

The *Frzb* proteins and the protein *Cerberus* are examples of secreted proteins that purportedly inhibit wnt signaling (Brown, *Curr. Opinion Cell Biol.*, 10:182-187 [1998).

PCT WO 98/35043, published 13 August 1998 describes human SDF-5 proteins which are purportedly useful in regulating the binding of *wnt* polypeptides to their receptors.

PCT WO 98/23730, published 4 June 1998, describes transfecting tumors cells with *wnt*-5a to purportedly decrease tumorigenicity. *Wnt*-5a purportedly is an antagonist of other *wnts*.

In view of the devastating effects of cancer, there is a need in the art to identify additional genes that may serve as antagonists of proteins involved in cell transformation.

Accordingly, it is an object of this invention to provide nucleic acid molecules and

polypeptides that may be useful as anti-cancer compounds.

It is a further object to provide methods of altering the level of expression and/or activity of such polypeptides in the human body.

Other related objects will readily be apparent from a reading of this disclosure.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides an isolated nucleic acid molecule encoding a biologically active DKR polypeptide selected from the group consisting of:

(a) the nucleic acid molecule comprising SEQ ID NO:1;

(b) the nucleic acid molecule comprising SEQ ID NO:2;

(c) the nucleic acid molecule comprising SEQ ID NO:3;

(d) the nucleic acid molecule comprising SEQ ID NO:4;

(e) the nucleic acid molecule comprising SEQ ID NO:5;

(f) the nucleic acid molecule comprising SEQ ID NO:6;

(g) the nucleic acid molecule comprising SEQ ID NO:7;

(h) the nucleic acid molecule comprising SEQ ID NO:75;

(i) the nucleic acid molecule comprising SEQ ID NO:76;

(j) the nucleic acid molecule comprising SEQ ID NO:77;

(k) the nucleic acid molecule comprising SEQ ID NO:78;

(l) the nucleic acid molecule encoding the polypeptide of SEQ ID NO:8;

(m) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:9;

(n) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:10, or a biologically active fragment thereof;

(o) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:11, or a biologically active fragment thereof;

(p) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:12, or a biologically active fragment thereof;

(q) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:13, or a biologically active fragment thereof;

(r) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:14, or a biologically active fragment thereof;

(s) a nucleic acid molecule that encodes a polypeptide that is at least 85 percent identical to the polypeptide of SEQ ID NOs: 10, 11, 12, 13, or 14;

(t) a nucleic acid molecule that encodes a biologically active DKR polypeptide that has 1-100 amino acid substitutions and/or deletions as compared with the polypeptide of any of SEQ ID NOs:8, 9, 10, 11, 12, 13, or 14; and

(u) a nucleic acid molecule that hybridizes under conditions of high stringency to any of (c), (d), (e), (f), (g), (h), (i), (k), (l), (m), (n), (o), (p), (q), (r), (s), and (t) above.

In another embodiment, the invention provides an isolated nucleic acid molecule that is the complement of any of the nucleic acid molecules above.

In yet another embodiment, the invention provides an isolated nucleic acid molecule encoding a biologically active DKR polypeptide selected from the group of: amino acids 16-350, 21-350, 22-350, 23-350, 33-350, or 42-350, 21-145, 40-145, 40-150, 45-145, 45-145, 145-290, 150-290, 300-350, or 310-350 of SEQ ID NO:9; amino acids 15-266, 24-266, or 32-266 of SEQ ID NO:10; amino acids 17-259, 26-259, or 34-359 of SEQ ID NO:12; and amino acids 19-224, 20-224, 21-224, or 22-224 of SEQ ID NO:14.

In other embodiments, the invention provides vectors comprising the nucleic acid molecules, and host cells comprising the vectors.

In still another embodiment, the invention provides a process for producing a biologically active DKR polypeptide comprising the steps of:

(a) expressing a polypeptide encoded by any of the nucleic acid molecules herein in a suitable host; and

(b) isolating the polypeptide.

In still one other embodiment, the invention provides a biologically active DKR polypeptide selected from the group consisting of:

(a) the polypeptide of SEQ ID NO:8;

(b) the polypeptide of SEQ ID NO:9;

(c) the polypeptide of SEQ ID NO:10;

(d) the polypeptide of SEQ ID NO:11;

(e) the polypeptide of SEQ ID NO:12;

(f) the polypeptide of SEQ ID NO:13;

(g) the polypeptide of SEQ ID NO:14;

(h) a polypeptide that has 1-100 amino acid substitutions or deletions as compared with the polypeptide of any of (a)-(g) above; and

(i) a polypeptide that is at least 85 percent identical to any of the polypeptides of (c)-(h) above.

In still one other embodiment, the invention provides the following polypeptides: a polypeptide that is amino acids 16-350, 21-350, 22-350, 23-350, 33-350, or 42-350, 21-145, 40-145, 40-150, 45-145, 45-145, 145-290, 145-300, 145-350, 150-290, 300-350, or 310-350 of Figure 9, a polypeptide that is amino acids 15, 266, 24-266, or 32-266 of Figure 10, a polypeptide that is amino acids 17-259, 26-259, or 34-259 of Figure 12, and a polypeptide that is amino acids 19-224, 20-224, 21-224, or 22-224 of Figure 14.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (SEQ ID NO:1) depicts the cDNA sequence of mouse DKR-3.

Figure 2 (SEQ ID NO:2) depicts the cDNA sequence of human DKR-3.

Figure 3 (SEQ ID NO:3) depicts the DNA sequence of human DKR-1.

Figure 4 (SEQ ID NO:4) depicts the cDNA sequence of mouse DKR-2.

Figure 5 (SEQ ID NO:5) depicts the cDNA sequence of human DKR-2.

Figure 6 (SEQ ID NO:6) depicts the cDNA sequence of human DKR-2a, a splice variant of the DKR-2 gene.

Figure 7 (SEQ ID NO:7) depicts the cDNA sequence of human DKR-4.

Figure 8 (SEQ ID NO:8) depicts the amino acid sequence of mouse DKR-3 as translated from the corresponding cDNA.

Figure 9 (SEQ ID NO:9) depicts the amino acid sequence of human DKR-3 as translated from the corresponding cDNA.

Figure 10 (SEQ ID NO:10) depicts the amino acid sequence of human DKR-1 as translated from the corresponding cDNA.

Figure 11 (SEQ ID NO:11) depicts the amino acid sequence of mouse DKR-2 as translated from the corresponding cDNA.

Figure 12 (SEQ ID NO:12) depicts the amino acid sequence of human DKR-2 as translated from the corresponding cDNA.

Figure 13 (SEQ ID NO:13) depicts the amino acid sequence of human DKR-2a as translated from the corresponding cDNA.

Figure 14 (SEQ ID NO:14) depicts the amino acid sequence of human DKR-4 as translated from the corresponding cDNA.

Figures 15A-15D are photographs of Northern blots which were probed with human DKR-3. Figure 15A shows the transcript level of DKR-3 in various human normal (Lanes 1-2) and immortal (Lanes 3-4) cell lines, and in human estrogen receptor plus ("ER+"; Lanes 5-9) and estrogen receptor minus ("ER-"; Lanes 10-16) breast cancer cell lines. Figure 15B shows the transcript level of human DKR-3 in human normal lung cells (Lane 1), and in various human non-small cell lung cancer ("NSCLC"; Lanes 2-9) and small cell lung cancer ("SCLC"; Lanes 10-15) cell lines. Figure 15C shows the amount of transcript of human DKR-3 in five glioblastoma cell lines; three of these lines (SNB-19, U-87MG, and U-373MG) are capable of forming tumors in nude mice, while the other two lines (Hs 683 and A 172) are not. Figure 15D shows the transcript level of human DKR-3 in human immortal (non-cancerous) and normal cervical cells, and in human cervical cancer cell lines (indicated as "tumor cells").

Figure 16 is a photograph of SDS gel electrophoresis. The contents of the lanes are set forth in the Examples herein.

Figure 17 is a photograph of SDS gel electrophoresis. The contents of the lanes are set forth in the Examples herein.

Figure 18 is a photograph of SDS gel electrophoresis. The contents of the lanes are set forth in the Examples herein.

Figure 19 is a photograph of SDS gel electrophoresis. The contents of the lanes are set forth in the Examples herein.

Figure 20 is a photograph of SDS gel electrophoresis. The contents of the lanes are set forth in the Examples herein.

Figure 21 is a photograph of a Western blot. Contents of the Lanes are indicated in the Examples herein.

Figure 22 (SEQ ID NO:75) is a nucleic acid sequence of human DKR-1 with codons optimized for expression in *E. coli*.

Figure 23 (SEQ ID NO:76) is a nucleic acid sequence of human DKR-2 with codons optimized for expression in *E. coli*.

Figure 24 SEQ ID NO:77) is a nucleic acid sequence of human DKR-3 with codons optimized for expression in *E. coli*.

Figure 25 (SEQ ID NO:78) is a nucleic acid sequence of human DKR-4 with codons optimized for expression in *E. coli*.

DETAILED DESCRIPTION OF THE INVENTION

Included in the scope of this invention are *DKR* polypeptides such as the polypeptides of SEQ ID

NOs:8-14, and related biologically active polypeptide fragments, variants, and derivatives thereof.

Also included within the scope of the present invention are nucleic acid molecules that encode *DKR* polypeptides such as the nucleic acid molecules of SEQ ID Nos:1-7.

Additionally included within the scope of the present invention are non-human mammals such as mice, rats, rabbits, goats, or sheep in which the gene (or genes) encoding a native *DKR* polypeptide has (have) been disrupted ("knocked out") such that the level of expression of this gene or genes is (are) significantly decreased or completely abolished. Such mammals may be prepared using techniques and methods such as those described in U.S. Patent No. 5,557,032. The present invention further includes non-human mammals such as mice, rats, rabbits, goats, or sheep in which the gene (or genes) encoding *DKR* polypeptides in which either the native form of the gene(s) for that mammal or a heterologous *DKR* polypeptide gene(s) is (are) over expressed by the mammal, thereby creating a "transgenic" mammal. Such transgenic mammals may be prepared using well known methods such as those described in U.S. Patent No 5,489,743 and PCT patent application no. WO94/28122, published 8 December 1994. The present invention further includes non-human mammals in which the promoter for one or more of the *DKR* polypeptides of the present invention is either activated or inactivated (using homologous recombination methods as described below) to alter the level of expression of one or more of the native *DKR* polypeptides.

The *DKR* polypeptides of the present invention are expected to have utility as anti-cancer therapeutics for those cancers such as mammary tumors,

stem cell tumors, or other cancers in which the *wnt* and/or sonic hedgehog (shh) signal transduction pathways are activated. Specific *wnt* members can transform mammary tissue (Hunter, supra) and are abnormally expressed in many human tumors (Huguet, *Cancer Res.*, 54:2615-2621 [1994]; Dale, *Cancer Res.*, 56:4320-4323 [1996]; see also PCT WO 97/39357). Such activity is expected in view of data presented herein in which the level of DKR-3 transcript is decreased or not detectable at all in many cancer cell lines as compared to similar normal cell lines. Further, such activity is expected in view of the relationship of the genes and polypeptides of the present invention to the gene *dickkopf-1* (which, as mentioned above, is purportedly a potent antagonist of *wnt*-8). DKR-1, a novel gene of the present invention, is a human ortholog of *dkk-1*. DKR-2, DKR-3, and DKR-4, all novel genes of the present invention, are each related to DKR-1 by their cysteine pattern. In particular, these *DKR* polypeptides may be of use for treatment of stem cell tumors, mammary tumors, and other cancers in which *wnt* genes are expressed, and in cancers where *wnt* and/or shh signaling is activated..

The *DKR* polypeptides of the present invention may also be administered as agents that can induce and/or enhance tissue differentiation, such as bone formation, cartilage formation, muscle tissue formation, nerve tissue formation, and hematopoietic cell formation. Such activities are expected in view of the fact that a) Xenopus dkk-1 purportedly promotes head induction, heart formation, and differentiation or the developing CNS (Glinka, supra); and b) certain *wnt* polypeptides appear to function in embryo development (Cadigan, *Genes and Devel.*, 11:3286-3305 [1997]), specifically development of the pituitary (Treier,

Genes and Devel., 12:1691-1704 [1998]), myogenesis (Munsterberg *et al.*, *Genes and Devel.*, 9:2911-2922 [1995]), osteogenesis (PCT WO 95/17416; PCT WO98/16641), kidney development (Stark *et al.*, *Nature* 372:679-683 [1994]), development of the CNS (Dickinson *et al.*, Development, 120:1453-1471 [1994]), and hematopoiesis (PCT WO 98/06747). Thus, addition of certain *DKR* polypeptides in such cell cultures or tissues may serve to modify the activity of various *wnt* polypeptides in cellular differentiation processes.

The *DKR* polypeptides herein may be used in either an *in vivo* manner or an *ex vivo* manner for such applications. For example, one or more of the *DKR* polypeptides of the present invention may be added to a culture of cartilage tissue or nerve tissue, or hematopoietic stem cells, either alone, or in combination with other growth factors and/or other tissue differentiation factors, so as to induce or enhance the regeneration of such tissues. Alternatively, such *DKR* polypeptides of the present invention may, for example, be injected directly into a joint in need of cartilage, into the spinal cord where the cord has been damaged, into damaged brain tissue, or into bone marrow to enhance hematopoiesis.

The term "*DKR* polypeptides" as used herein refers to any protein or polypeptide having the properties described herein for *DKR* polypeptides. The *DKR* polypeptides may or may not have amino terminal methionines, depending on the manner in which they are prepared. By way of illustration, *DKR* polypeptides refers to (1) a biologically active polypeptide encoded by any of the *DKR* polypeptides nucleic acid molecules as defined in any of items (a)-(f) below; (2) naturally occurring allelic variants and synthetic variants of any of *DKR* polypeptide in which one or more amino acid

substitutions, deletions, and/or insertions are present as compared to the *DKR* polypeptides of SEQ ID NOs:8-14, and/or (3) biologically active polypeptides, or fragments or variants thereof, that have been chemically modified.

As used herein, the term "*DKR polypeptide* fragment" refers to a peptide or polypeptide that is less than the full length amino acid sequence of a naturally occurring *DKR polypeptide* but has the biological activity of any of the *DKR* polypeptides provided herein. Such a fragment may be truncated at the amino terminus, the carboxy terminus, and/or internally (such as by natural splicing), and may be a variant or a derivative of any of the *DKR* polypeptides. Such *DKR* polypeptides fragments may be prepared with or without an amino terminal methionine. In addition, *DKR* polypeptides fragments can be naturally occurring fragments such as *DKR polypeptide* splice variants (SEQ ID NO:13), other splice variants, and fragments resulting from naturally occurring *in vivo* protease activity. Preferred DKR polypeptide fragments include amino acids 16-350, 21-350, 22-350, 23-350, 33-350, 42-350, 21-145, 40-145, 40-150, 45-145, 145-290, 145-300, 145-350, 150-290, 300-350, and 310-350, of SEQ ID NO:9; amino acids 15-266, 24-266, or 32-266 of SEQ ID NO:10; amino acids 17-259, 26-259, or 34-359 of SEQ ID NO:12; and amino acids 19-224, 20-224, 21-224, or 22-224 of SEQ ID NO:14.

As used herein, the term "*DKR polypeptide* variants" refers to *DKR* polypeptides whose amino acid sequences contain one or more amino acid sequence substitutions, deletions, and/or insertions as compared to the *DKR* polypeptides amino acid sequences set forth in SEQ ID NOS:8-14. Such *DKR* polypeptides variants can be prepared from the corresponding *DKR* polypeptides

nucleic acid molecule variants, which have a DNA sequence that varies accordingly from the DNA sequences for wild type *DKR* polypeptides as set forth in SEQ ID NOS:7-14. Preferred variants of the human *DKR* polypeptides include alanine substitutions at one or more of amino acid positions. Other preferred substitutions include conservative substitutions at the amino acid positions indicated in the Examples herein, as well as those encoded by DKR nucleic acid molecules as described below.

As used herein, the term "*DKR* polypeptide derivatives" refers to *DKR* polypeptides, variants, or fragments thereof, that have been chemically modified, as for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, and/or other such molecules, where the molecule or molecules are not naturally attached to wild-type *DKR* polypeptides.

As used herein, the terms "biologically active *DKR* polypeptides", "biologically active *DKR* polypeptide fragments", "biologically active *DKR* polypeptide variants", and "biologically active *DKR* polypeptide derivatives" refer to *DKR* polypeptides that have the ability to decrease cancer cell proliferation in the Anchorage Independent Growth Assay of Example 12 herein, or in the *In Vivo* Tumor Assay of Example 13 herein, or in both assays.

As used herein, the term " *DKR* polypeptide nucleic acids" when used to describe a nucleic acid molecule refers to a nucleic acid molecule or fragment thereof that (a) has the nucleotide sequence as set forth in any of SEQ ID NOs:1-7; (b) has a nucleic acid sequence encoding a polypeptide that is at least 85 percent identical, but may be greater than 85 percent, *i.e.*,86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 percent identical to the polypeptide encoded

by any of SEQ ID NOS:10-14; (c) is a naturally occurring allelic variant or alternate splice variant of (a) or (b); (d) is a nucleic acid variant of (a)-(c) produced as provided for herein;(e) has a sequence that is complementary to (a)-(d); (f) hybridizes to any of (a)-(e) under conditions of high stringency and/or (g) has a nucleic acid sequence encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or up to 100 amino acid substitutions and/or deletions of any mature DKR polypeptide(i.e., a DKR polypeptide with its endogenous signal peptide removed).

Percent sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. By way of example, using a computer algorithm such as GAP (Genetic Computer Group, University of Wisconsin, Madison, WI), the two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3 X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff *et al.*, in: *Atlas of Protein Sequence and Structure*, vol. 5, supp.3 [1978] for the PAM250 comparison matrix; see Henikoff et al., *Proc. Natl. Acad. Sci USA*,

89:10915-10919 [1992] for the BLOSUM 62 comparison matrix) is also used by the algorithm. The percent identity is then calculated by the algorithm by determining the percent identity as follows:

$$\frac{\text{Total number of identical matches in the matched span}}{\text{[length of the longer sequence within the matched span] + [number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Polypeptides that are at least 85 percent identical will typically have one or more amino acid substitutions, deletions, and/or insertions as compared with any of the wild type *DKR* polypeptides. Usually, the substitutions of the native residue will be either alanine, or a conservative amino acid so as to have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Conservative substitutions are set forth in Table I below.

Table I

Conservative Amino Acid Substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Uncharged Polar:	glutamine
	asparagine
	serine
	threonine
	tyrosine
Non-Polar:	phenylalanine
	tryptophan
	cysteine
	glycine
	alanine
	valine
	proline
	methionine
	leucine
	isoleucine

The term " conditions of high stringency" refers to hybridization and washing under conditions that permit binding of a nucleic acid molecule used for screening, such as an oligonucleotide probe or cDNA molecule probe, to highly homologous sequences. An exemplary high stringency wash solution is 0.2 X SSC and 0.1 percent SDS used at a temperature of between 50°C-65°C.

Where oligonucleotide probes are used to screen cDNA or genomic libraries, one of the following two high stringency solution may be used. The first of

these is 6 X SSC with 0.05 percent sodium pyrophosphate at a temperature of 35°C-62°C, depending on the length of the oligonucleotide probe. For example, 14 base pair probes are washed at 35-40°C, 17 base pair probes are washed at 45-50°C, 20 base pair probes are washed at 52-57°C, and 23 base pair probes are washed at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second high stringency solution utilizes tetramethylammonium chloride (TMAC) for washing oligonucleotide probes. One stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2 percent SDS. The washing temperature using this solution is a function of the length of the probe. For example, a 17 base pair probe is washed at about 45-50°C.

As used herein, the terms "effective amount" and "therapeutically effective amount" refer to the amount of a *DKR* polypeptide necessary to support one or more biological activities of the *DKR* polypeptides as set forth above.

A full-length *DKR* polypeptide or fragment thereof can be prepared using well known recombinant DNA technology methods such as those set forth in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and/or Ausubel *et al.*, eds., (*Current Protocols in Molecular Biology*, Green Publishers Inc. and Wiley and Sons, NY [1994]). A gene or cDNA encoding a *DKR* polypeptide or fragment thereof may be obtained for example by screening a genomic or cDNA library, or by PCR amplification. Probes or primers useful for screening the library can be generated based on sequence information for other known genes or gene fragments from the same or a related family of genes, such as, for example, conserved motifs

found in other *DKR* polypeptides such as the cysteine pattern. In addition, where a gene encoding *DKR* polypeptide has been identified from one species, all or a portion of that gene may be used as a probe to identify homologous genes from other species. The probes or primers may be used to screen cDNA libraries from various tissue sources believed to express the *DKR* gene. Typically, conditions of high stringency will be employed for screening to minimize the number of false positives obtained from the screen.

Another means to prepare a gene encoding a *DKR* polypeptide or fragment thereof is to employ chemical synthesis using methods well known to the skilled artisan such as those described by Engels *et al.*(*Angew. Chem. Intl. Ed.*, 28:716-734 [1989]). These methods include, *inter alia*, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the *DKR* polypeptide will be several hundred nucleotides in length. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form the full length *DKR* polypeptide. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the *DKR* polypeptide, depending on whether the polypeptide produced in the host cell is designed to be secreted from that cell.

In some cases, it may be desirable to prepare nucleic acid and/or amino acid variants of the naturally occurring *DKR* polypeptides. Nucleic acid variants may be produced using site directed

mutagenesis, PCR amplification, or other appropriate methods, where the primer(s) have the desired point mutations (see Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels *et al.*, *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well. Preferred nucleic acid variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to produce the *DKR* polypeptide(s). Such "codon optimization" can be determined via computer algorithers which incorporate codon frequency tables such as "Ecohigh. Cod" for codon preference of highly expressed bacterial genes as provided by the University of Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI. Other useful codon frequency tables include "Celegans_high.cod", "Celegans_low.cod", "Drosophila_high.cod", "Human_high.cod", "Maize_high.cod", and "Yeast_high.cod". Other preferred variants are those encoding conservative amino acid changes as described above (*e.g.*, wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s), or those designed to delete an existing glycosylation and/or phosphorylation site(s).

The gene, cDNA, or fragment thereof encoding the *DKR* polypeptide can be inserted into an appropriate expression or amplification vector using standard ligation techniques. The vector is typically selected to be functional in the particular host cell employed

(*i.e.*, the vector is compatible with the host cell machinery such that amplification of the gene and/or expression of the gene can occur). The gene, cDNA or fragment thereof encoding the *DKR* polypeptide may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic host cells. Selection of the host cell will depend in part on whether the *DKR* polypeptide or fragment thereof is to be glycosylated and/or phosphorylated. If so, yeast, insect, or mammalian host cells are preferable.

Typically, the vectors used in any of the host cells will contain 5' flanking sequence (also referred to as a "promoter") and other regulatory elements as well such as an enhancer(s), an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these elements is discussed below. Optionally, the vector may contain a "tag" sequence, *i.e.*, an oligonucleotide molecule located at the 5' or 3' end of the *DKR* polypeptide coding sequence; the oligonucleotide molecule encodes polyHis (such as hexaHis), or other "tag" such as FLAG, HA (hemaglutinin Influenza virus) or *myc* for which commercially available antibodies exist. This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as means for affinity purification of the *DKR* polypeptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified *DKR*

polypeptide by various means such as using certain peptidases.

The human immunoglobulin hinge and Fc region could be fused at either the N-terminus or C-terminus of the DKR polypeptides by one skilled in the art. The subsequent Fc-fusion protein could be purified by use of a Protein A affinity column. Fc is known to exhibit a long pharmacokinetic half-life *in vivo* and proteins fused to Fc have been found to exhibit a substantially greater half-life *in vivo* than the unfused counterpart. Also, fusion to the Fc region allows for dimerization/multimerization of the molecule that may be useful for the bioactivity of some molecules.

The 5' flanking sequence may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than the host cell species or strain), hybrid (*i.e.*, a combination of 5' flanking sequences from more than one source), synthetic, or it may be the native *DKR* polypeptides gene 5' flanking sequence. As such, the source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by, the host cell machinery.

The 5' flanking sequences useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, 5' flanking sequences useful herein other than the *DKR* gene flanking sequence will have been previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the full nucleotide sequence of the 5' flanking sequence may be

known. Here, the 5' flanking sequence may be synthesized using the methods described above for nucleic acid synthesis or cloning.

Where all or only a portion of the 5' flanking sequence is known, it may be obtained using PCR and/or by screening a genomic library with suitable oligonucleotide and/or 5' flanking sequence fragments from the same or another species.

Where the 5' flanking sequence is not known, a fragment of DNA containing a 5' flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion using one or more carefully selected enzymes to isolate the proper DNA fragment. After digestion, the desired fragment may be isolated by agarose gel purification, Qiagen® column or other methods known to the skilled artisan. Selection of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

The origin of replication element is typically a part of prokaryotic expression vectors purchased commercially, and aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of the *DKR* polypeptide. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector.

The transcription termination element is typically located 3' of the end of the *DKR* polypeptide coding sequence and serves to terminate transcription of the *DKR* polypeptide. Usually, the transcription termination element in prokaryotic cells is a G-C rich

fragment followed by a poly T sequence. While the element is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described above.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene.

The ribosome binding element, commonly called the Shine-Dalgarno sequence (prokaryotes) or the Kozak sequence (eukaryotes), is usually necessary for translation initiation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the *DKR* polypeptide to be synthesized. The Shine-Dalgarno sequence is varied but is typically a polypurine (*i.e.*, having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

In those cases where it is desirable for *DKR* polypeptide to be secreted from the host cell, a signal sequence may be used to direct the *DKR* polypeptide out of the host cell where it is synthesized, and the carboxy-terminal part of the protein may be deleted in order to prevent membrane anchoring. Typically, the

signal sequence is positioned in the coding region of the *DKR* gene or cDNA, or directly at the 5' end of the *DKR* gene coding region. Many signal sequences have been identified, and any of them that are functional in the selected host cell may be used in conjunction with the *DKR* gene or cDNA. Therefore, the signal sequence may be homologous or heterologous to the *DKR* gene or cDNA, and may be homologous or heterologous to the *DKR* polypeptides gene or cDNA. Additionally, the signal sequence may be chemically synthesized using methods set forth above.

In most cases, secretion of the polypeptide from the host cell via the presence of a signal peptide will result in the removal of the amino terminal methionine from the polypeptide.

In many cases, transcription of the *DKR* gene or cDNA is increased by the presence of one or more introns in the vector; this is particularly true where the *DKR* polypeptide is produced in eukaryotic host cells, especially mammalian host cells. The introns used may be naturally occurring within the *DKR* gene, especially where the gene used is a full length genomic sequence or a fragment thereof. Where the intron is not naturally occurring within the gene (as for most cDNAs), the intron(s) may be obtained from another source. The position of the intron with respect to the 5' flanking sequence and the *DKR* gene is generally important, as the intron must be transcribed to be effective. As such, where the *DKR* gene inserted into the expression vector is a cDNA molecule , the preferred position for the intron is 3' to the transcription start site, and 5' to the polyA transcription termination sequence. Preferably for *DKR* cDNA, the intron or introns will be located on one side or the other (*i.e.*, 5' or 3') of the cDNA such that it

does not interrupt the this coding sequence. Any intron from any source, including any viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell(s) into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector.

Where one or more of the elements set forth above are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are comparable to the methods set forth above (*i.e.*, synthesis of the DNA, library screening, and the like).

The final vectors used to practice this invention are typically constructed from a starting vectors such as a commercially available vector. Such vectors may or may not contain some of the elements to be included in the completed vector. If none of the desired elements are present in the starting vector, each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to "blunt" the ends to be ligated together in order to obtain a satisfactory ligation. Blunting is accomplished by first filling in "sticky ends" using Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook *et al.*, *supra*.

Alternatively, two or more of the elements to be inserted into the vector may first be ligated

together (if they are to be positioned adjacent to each other) and then ligated into the vector.

One other method for constructing the vector to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors will be generated due to improper ligation or insertion of the elements, however the functional vector may be identified and selected by restriction endonuclease digestion.

Preferred vectors for practicing this invention are those which are compatible with bacterial, insect, and mammalian host cells. Such vectors include, *inter alia*, pCRII, pCR3, and pcDNA3.1 (Invitrogen Company, San Diego, CA), pBSII (Stratagene Company, La Jolla, CA), pET15b (Novagen, Madison, WI), pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2 (Clontech, Palo Alto, CA), pETL (BlueBacII; Invitrogen), and pFastBacDual (Gibco/BRL, Grand Island, NY).

After the vector has been constructed and a nucleic acid molecule encoding full length or truncated *DKR* polypeptide has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression.

Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, can synthesize *DKR* polypeptide which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). After collection, the *DKR* polypeptide can be purified using

methods such as molecular sieve chromatography, affinity chromatography, and the like.

Selection of the host cell for *DKR* polypeptide production will depend in part on whether the *DKR* polypeptide is to be glycosylated or phosphorylated (in which case eukaryotic host cells are preferred), and the manner in which the host cell is able to "fold" the protein into its native tertiary structure (*e.g.*, proper orientation of disulfide bridges, etc.) such that biologically active protein is prepared by the *DKR* polypeptide that has biological activity, the *DKR* polypeptide may be "folded" after synthesis using appropriate chemical conditions as discussed below.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO), human embryonic kidney (HEK) 293 or 293T cells, or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5α, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention.

Additionally, where desired, insect cell systems may be utilized in the methods of the present invention. Such systems are described for example in Kitts *et al.* (*Biotechniques*, 14:810-817 [1993]), Lucklow (*Curr. Opin. Biotechnol.*, 4:564-572 [1993])and Lucklow *et al.* (*J. Virol.*, 67:4566-4579 [1993]). Preferred insect cells are Sf-9 and Hi5 (Invitrogen, Carlsbad, CA).

Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such methods as calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, *supra*.

The host cells containing the vector (*i.e.*, transformed or transfected) may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media

for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary.

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of *DKR* polypeptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If the *DKR* polypeptide has been designed to be secreted from the host cells, the majority of polypeptide may be found in the cell culture medium. Polypeptides prepared in this way will typically not possess an amino terminal methionine, as it is removed during secretion from the cell. If however, the *DKR* polypeptide is not secreted from the host cells, it will be present in the cytoplasm and/or the nucleus (for eukaryotic host cells) or in the cytosol (for gram negative bacteria host cells) and may have an amino terminal methionine.

For *DKR* polypeptide situated in the host cell cytoplasm and/or nucleus, the host cells are typically first disrupted mechanically or with detergent to release the intra-cellular contents into a buffered solution. *DKR* polypeptide can then be isolated from this solution.

Purification of *DKR* polypeptide from solution can be accomplished using a variety of techniques. If the polypeptide has been synthesized such that it contains a tag such as Hexahistidine (*DKR* polypeptide/hexaHis) or other small peptide such as FLAG (Eastman Kodak Co., New Haven, CT) or *myc* (Invitrogen, Carlsbad, CA) at either its carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide directly (*i.e.*, a monoclonal antibody specifically recognizing *DKR* polypeptide). For example, polyhistidine binds with great affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen® nickel columns) can be used for purification of *DKR* polypeptide/polyHis. (See for example, Ausubel *et al.*, eds., *Current Protocols in Molecular Biology*, Section 10.11.8, John Wiley & Sons, New York [1993]).

Where the *DKR* polypeptide is prepared without a tag attached, and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of

these techniques may be combined to achieve increased purity.

If it is anticipated that the *DKR* polypeptide will be found primarily intracellularly, the intracellular material (including inclusion bodies for gram-negative bacteria) can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm/cytoplasm by French press, homogenization, and/or sonication followed by centrifugation.

If the *DKR* polypeptide has formed inclusion bodies in the cytosol, the inclusion bodies can often bind to the inner and/or outer cellular membranes and thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated at pH extremes or with chaotropic agent such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris carboxyethyl phosphine at acid pH to release, break apart, and solubilize the inclusion bodies. The *DKR* polypeptide in its now soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If it is desired to isolate the *DKR* polypeptide, isolation may be accomplished using standard methods such as those set forth below and in Marston *et al.* (*Meth. Enz.*, 182:264-275 [1990]). In some cases, the DKR polypeptide may not be biologically active upon isolation. Various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity. Such methods include

exposing the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization but usually at a lower concentration and is not necessarily the same chaotrope as used for the solubilization. In most cases the refolding/oxidation solution will also contain a reducing agent or the reducing agent plus its' oxidized form in a specific ratio to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of the protein's cysteine bridge(s). Some of the commonly used redox couples include cysteine/cystamine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol(DTT)/dithiane DTT, 2-mercaptoethanol(bME)/dithio-b(ME). In many instances a cosolvent is necessary to increase the efficiency of the refolding and the more common reagents used for this purpose include glycerol, polyethylene glycol of various molecular weights, and arginine.

If *DKR* polypeptide inclusion bodies are not formed to a significant degree in the host cell, the *DKR* polypeptide will be found primarily in the supernatant after centrifugation of the cell homogenate, and the *DKR* polypeptide can be isolated from the supernatant using methods such as those set forth below.

In those situations where it is preferable to partially or completely isolate the *DKR* polypeptide, purification can be accomplished using standard methods well known to the skilled artisan. Such methods include, without limitation, separation by electrophoresis followed by electroelution,

various types of chromatography (immunoaffinity, molecular sieve, and/or ion exchange), and/or high pressure liquid chromatography. In some cases, it may be preferable to use more than one of these methods for complete purification.

In addition to preparing and purifying *DKR* polypeptide using recombinant DNA techniques, the *DKR* polypeptides, fragments, and/or derivatives thereof may be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using techniques known in the art such as those set forth by Merrifield *et al.*, (*J. Am. Chem. Soc.*, 85:2149 [1963]), Houghten *et al.* (*Proc Natl Acad. Sci. USA*, 82:5132 [1985]), and Stewart and Young (Solid Phase Peptide Synthesis, Pierce Chemical Co., Rockford, IL [1984]). Such polypeptides may be synthesized with or without a methionine on the amino terminus. Chemically synthesized *DKR* polypeptides or fragments may be oxidized using methods set forth in these references to form disulfide bridges. The *DKR* polypeptides or fragments are expected to have biological activity comparable to *DKR* polypeptides produced recombinantly or purified from natural sources, and thus may be used interchangeably with recombinant or natural *DKR* polypeptide.

Chemically modified *DKR* polypeptide compositions in which *DKR* polypeptide is linked to a polymer are included within the scope of the present invention. The polymer selected is typically water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for

alkylation, so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of *DKR* polypeptide polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

The water soluble polymer or mixture thereof may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol.

For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Patent No. 5,252,714).

Pegylation of *DKR* polypeptides may be carried out by any of the pegylation reactions known in the art, as described for example in the following references: *Focus on Growth Factors* 3: 4-10 (1992); EP 0 154 316; and EP 0 401 384. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described below.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable conditions used to react a biologically active substance with an activated polymer molecule. Methods for preparing pegylated *DKR* polypeptides will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby *DKR polypeptide* becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/ polypeptides include those described herein for *DKR* polypeptides molecules. However, the polymer/ *DKR* polypeptides molecules disclosed herein may have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to the non-derivatized molecules.

The *DKR* polypeptides, fragments thereof, variants, and derivatives, may be employed alone, together, or in combination with other pharmaceutical compositions. The *DKR* polypeptides, fragments, variants, and derivatives may be used in combination

with cytokines, growth factors, antibiotics, anti-inflammatories, and/or chemotherapeutic agents as is appropriate for the indication being treated.

DKR nucleic acid molecules, fragments, and/or derivatives that do not themselves encode polypeptides that are active in activity assays may be useful as hybridization probes in diagnostic assays to test, either qualitatively or quantitatively, for the presence of *DKR* DNA or corresponding RNA in mammalian tissue or bodily fluid samples.

DKR polypeptide fragments, variants, and/or derivatives that are not themselves active in activity assays may be useful for preparing antibodies that recognize *DKR* polypeptides.

The *DKR* polypeptides, fragments, variants, and/or derivatives may be used to prepare antibodies using standard methods. Thus, antibodies that react with the *DKR* polypeptides, as well as reactive fragments of such antibodies, are also contemplated as within the scope of the present invention. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific. Typically, the antibody or fragment thereof will either be of human origin, or will be "humanized", *i.e.*, prepared so as to prevent or minimize an immune reaction to the antibody when administered to a patient. The antibody fragment may be any fragment that is reactive with *DKR* polypeptides of the present invention, such as, F_{ab}, $F_{ab'}$, etc. Also provided by this invention are the hybridomas generated by presenting any *DKR* polypeptide or fragments thereof as an antigen to a selected mammal, followed by fusing cells (*e.g.*, spleen cells) of the mammal with certain cancer cells to create immortalized cell lines by known techniques. The methods employed to generate such cell lines and

antibodies directed against all or portions of a human *DKR* polypeptide of the present invention are also encompassed by this invention.

The antibodies may be used therapeutically, such as to inhibit binding of the *DKR* polypeptide to its binding partner. The antibodies may further be used for *in vivo* and *in vitro* diagnostic purposes, such as in labeled form to detect the presence of *DKR* polypeptide in a body fluid or cell sample.

Preferred antibodies are human antibodies, either polyclonal or monoclonal.

Therapeutic Compositions and Administration

Therapeutic compositions of *DKR* polypeptides are within the scope of the present invention. Such compositions may comprise a therapeutically effective amount of the polypeptide or fragments, variants, or derivatives in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, a *DKR* polypeptide therapeutic compound will be administered in the form of a composition comprising purified polypeptide, fragment, variant, or derivative in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (*e.g.*, sucrose). Other standard carriers, diluents, and excipients may be included as desired. Other exemplary compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor.

The *DKR* polypeptide compositions can be administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art.

Therapeutic formulations of *DKR* polypeptide compositions useful for practicing the present invention may be prepared for storage by mixing the selected composition having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences*, 18th Edition, A.R. Gennaro, ed., Mack Publishing Company [1990]) in the form of a lyophilized cake or an aqueous solution. Acceptable carriers, excipients or stabilizers are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, pluronics or polyethylene glycol (PEG).

An effective amount of the *DKR* polypeptide composition(s) to be employed therapeutically will depend, for example, upon the therapeutic objectives such as the indication for which the *DKR* polypeptide is being used, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage may range from about 0.1 μg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, a clinician will administer the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of *DKR* polypeptide) over time, or as a continuous infusion via implantation device or catheter.

As further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, the type of disorder under treatment, the age and general health of the recipient, will be able to ascertain proper dosing.

The *DKR* polypeptide composition to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using these methods may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration ordinarily will be stored in lyophilized form or in solution.

Therapeutic compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration of the composition is in accord with known methods, *e.g.* oral, injection or infusion by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems or implantation device which may optionally involve the use of a catheter. Where desired, the compositions may be administered continuously by infusion, bolus injection or by implantation device.

Alternatively or additionally, the composition may be administered locally via implantation into the affected area of a membrane, sponge, or other appropriate material on to which *DKR* polypeptide has been absorbed.

Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of *DKR* polypeptide may be directly through the device via bolus, or via continuous administration, or via catheter using continuous infusion.

DKR polypeptide may be administered in a sustained release formulation or preparation. Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al*, *Biopolymers*, 22: 547-556 [1983]), poly (2-

hydroxyethyl-methacrylate) (Langer *et al.*, *J. Biomed. Mater. Res.*, 15: 167-277 [1981] and Langer, *Chem. Tech.*, 12: 98-105 [1982]), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also may include liposomes, which can be prepared by any of several methods known in the art (*e.g.*, Eppstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82: 3688-3692 [1985]; EP 36,676; EP 88,046; EP 143,949).

In some cases, it may be desirable to use *DKR* polypeptide compositions in an *ex vivo* manner. Here, cells, tissues, or organs that have been removed from the patient are exposed to *DKR* polypeptide compositions after which the cells, tissues and/or organs are subsequently implanted back into the patient.

In other cases, *DKR* polypeptide may be delivered through implanting into patients certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the polypeptides, fragments, variants, or derivatives. Such cells may be animal or human cells, and may be derived from the patient's own tissue or from another source, either human or non-human. Optionally, the cells may be immortalized. However, in order to decrease the chance of an immunological response, it is preferred that the cells be encapsulated to avoid infiltration of surrounding tissues. The encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow release of the protein product(s) but prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

Methods used for membrane encapsulation of cells are familiar to the skilled artisan, and

preparation of encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, *e.g.*, U.S Patent Nos. 4,892,538; 5,011,472; and 5,106,627. A system for encapsulating living cells is described in PCT WO 91/10425 (Aebischer et al.). Techniques for formulating a variety of other sustained or controlled delivery means, such as liposome carriers, bio-erodible particles or beads, are also known to those in the art, and are described, for example, in U.S. Patent No. 5,653,975 (Baetge *et al.*, CytoTherapeutics, Inc.). The cells, with or without encapsulation, may be implanted into suitable body tissues or organs of the patient.

As discussed above, it may be desirable to treat isolated cell populations such as stem cells, lymphocytes, red blood cells, chondrocytes, neurons, and the like with one or more *DKR* polypeptides, variants, derivatives and/or fragments. This can be accomplished by exposing the isolated cells to the polypeptide, variant, derivative, or fragment directly, where it is in a form that is permeable to the cell membrane. Alternatively, gene therapy can be employed as described below.

One manner in which gene therapy can be applied is to use the *DKR* gene (either genomic DNA, cDNA, and/or synthetic DNA encoding a *DKR* polypeptide, or a fragment, variant, or derivative thereof) which may be operably linked to a constitutive or inducible promoter to form a "gene therapy DNA construct". The promoter may be homologous or heterologous to the endogenous *DKR* gene, provided that it is active in the cell or tissue type into which the construct will be inserted. Other components of the gene therapy DNA construct may optionally include, as required, DNA molecules designed for site-specific integration (*e.g.*,

endogenous flanking sequences useful for homologous recombination), tissue-specific promoter, enhancer(s) or silencer(s), DNA molecules capable of providing a selective advantage over the parent cell, DNA molecules useful as labels to identify transformed cells, negative selection systems, cell specific binding agents (as, for example, for cell targeting) cell-specific internalization factors, and transcription factors to enhance expression by a vector as well as factors to enable vector manufacture.

This gene therapy DNA construct can then be introduced into the patient's cells (either *ex vivo* or *in vivo*). One means for introducing the gene therapy DNA construct is via viral vectors. Suitable viral vectors typically used in gene therapy for delivery of gene therapy DNA constructs include, without limitation, adenovirus, adeno-assoicated virus, herpes simplex virus, lentivirus, papilloma virus, and retrovirus vectors. Some of these vectors, such as retroviral vectors, will deliver the gene therapy DNA construct to the chromosomal DNA of the patient's cells, and the gene therapy DNA construct can integrate into the chromosomal DNA; other vectors will function as episomes and the gene therapy DNA construct will remain in the cytoplasm. The use of gene therapy vectors is described, for example, in U.S. Patent Nos. 5,672,344 (30 September 1997; Kelly *et al.*, University of Michigan), 5,399,346 (21 March 1995; Anderson *et al.*, U.S Dept. Health and Human Services), 5,631,236 (20 May 1997; Woo *et al.*, Baylor College of Medicine), and 5,635,399 (3 June 1997; Kriegler *et al.*, Chiron Corp.).

Alternative means to deliver gene therapy DNA constructs to a patient's cells without the use of viral vectors include, without limitation, liposome-

mediated transfer, direct injection of naked DNA, receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation, and microparticle bombardment (*e.g.*, "gene gun"). See U.S. Patent Nos. 4,970,154 (13 November 1990; Chang, Baylor College of Medicine), WO 96/40958 (19 December 1996; Smith *et al.*, Baylor College of Medicine) 5,679,559 (21 October 1997; Kim *et al.*, University of Utah) 5,676,954 (14 October 1997; Brigham, Vanderbilt University), and 5,593,875 (14 January 1997; Wurm *et al.*, Genentech).

Another means to increase endogenous *DKR* polypeptide expression in a cell via gene therapy is to insert one or more enhancer elements into the *DKR* polypeptide promoter, where the enhancer element(s) can serve to increase transcriptional activity of the *DKR* polypeptides gene. The enhancer element(s) used will be selected based on the tissue in which one desires to activate the gene(s); enhancer elements known to confer promoter activation in that tissue will be selected. For example, if a *DKR* polypeptide is to be "turned on" in T-cells, the *lck* promoter enhancer element may be used. Here, the functional portion of the transcriptional element to be added may be inserted into a fragment of DNA containing the *DKR* polypeptide promoter (and optionally, vector, 5' and/or 3' flanking sequence, etc.) using standard cloning techniques. This construct, known as a "homologous recombination construct" can then be introduced into the desired cells either *ex vivo* or *in vivo*.

Gene therapy can be used to decrease *DKR* polypeptide expression by modifying the nucleotide sequence of the endogenous promoter(s). Such modification is typically accomplished via homologous recombination methods. For example, a DNA molecule containing all or a portion of the promoter of the *DKR*

gene(s) selected for inactivation can be engineered to remove and/or replace pieces of the promoter that regulate transcription. Here, the TATA box and/or the binding site of a transcriptional activator of the promoter may be deleted using standard molecular biology techniques; such deletion can inhibit promoter activity thereby repressing transcription of the corresponding *DKR* gene. Deletion of the TATA box or transcription activator binding site in the promoter may be accomplished by generating a DNA construct comprising all or the relevant portion of the *DKR* polypeptide promoter(s) (from the same or a related species as the *DKR* gene(s) to be regulated) in which one or more of the TATA box and/or transcriptional activator binding site nucleotides are mutated via substitution, deletion and/or insertion of one or more nucleotides such that the TATA box and/or activator binding site has decreased activity or is rendered completely inactive. This construct, which also will typically contain at least about 500 bases of DNA that correspond to the native (endogenous) 5′ and 3′ flanking regions of the promoter segment that has been modified, may be introduced into the appropriate cells (either *ex vivo* or *in vivo*) either directly or via a viral vector as described above. Typically, integration of the construct into the genomic DNA of the cells will be via homologous recombination, where the 5′ and 3′ flanking DNA sequences in the promoter construct can serve to help integrate the modified promoter region via hybridization to the endogenous chromosomal DNA.

Other gene therapy methods may also be employed where it is desirable to inhibit one or more *DKR* polypeptides. For example, antisense DNA or RNA molecules, which have a sequence that is complementary

to at least a portion of the selected *DKR* polypeptide gene(s) can be introduced into the cell. Typically, each such antisense molecule will be complementary to the start site (5' end) of each selected *DKR* gene. When the antisense molecule then hybridizes to the corresponding *DKR* polypeptides mRNA, translation of this mRNA is prevented.

Alternatively, gene therapy may be employed to create a dominant-negative inhibitor of one or more of the *DKR* polypeptides. In this situation, the DNA encoding a mutant full length or truncated polypeptide of each selected *DKR* polypeptide can be prepared and introduced into the cells of a patient using either viral or non-viral methods as described above. Each such mutant is typically designed to compete with endogenous polypeptide in its biological role.

Samples of the *E coli* cell lines GM121 and GM94 have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA, USA on DATE as accession numbers X and Y, respectively.

The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

EXAMPLES

Example 1: Cloning of the Mouse DKR-3 Gene

About 120 adult mice with an average body weight of about 18 grams were each injected intraperitoneally with a kainate solution (prepared as a stock solution of about 1 mg/ml kainate in sterile PBS) at a dose of about 25 mg kainate per kilogram body weight. About six hours after injection, the mice were sacrificed, and the hippocampus was dissected from each mouse. Total RNA was extracted from hippocampal tissue using the Trizol method (Gibco BRL, Grand Island, NY). The poly(A+) mRNA fraction was isolated from total RNA using Message Maker (Gibco BRL, Grand Island, NY) according to the manufacturer's recommended procedure. Hippocampal tissue was also obtained from control mice (which received an injection of PBS only), and poly(a+) mRNA was obtained from this tissue as well using the same procedures.

Two random primed cDNA libraries were prepared; one from the kainate-treated and one from the control poly (A+) mRNA using the Superscript® plasmid system (Gibco BRL, Gaithersburg, MD). A random cDNA primer containing an internal *NotI* restriction site was used to initiate first strand synthesis and had the following sequence:

GGAAGGAAAAAAGCGGCCGCAACANNNNNNNNN (SEQ ID NO:15)

where N is A, G, C, or T.

Both first strand cDNA synthesis and second strand cDNA synthesis were performed according to the manufacturer's recommended protocol. After second

strand synthesis, the reaction products were extracted with phenol:chloroform:isoamyl alcohol (in a volume ratio of 25:24:1), followed by ethanol precipitation. The double strand cDNA products were ligated using standard ligation procedures to the following double stranded oligonucleotide adapter (obtained from Gibco BRL, Grand Island, NY):

TCGACCCACGCGTCCG (SEQ ID NO:16)

GGGTGCGCAGGC (SEQ ID NO:17)

After ligation, the cDNA was digested to completion with *NotI*, and size fractionated on a 1 percent agarose gel. The cDNA products between about 250 and 800 base pairs were selected and purified from the gel using the Qiagen® gel extraction kit (Qiagen, Chatsworth, CA). The purified cDNA products were directionally ligated into the vector pYY41L (American Type Culture Collection, "ATCC"; 10801 University Blvd., Manassas, VA, USA; accession number 209636) which had been previously digested with *NotI* and *SalI*. The ligated cDNA was then introduced into electrocompetent ElectroMax® DH108 *E. coli* cells (Gibco-BRL, Grand Island, NY) via standard electroporation techniques. The library was then titered by a serial dilution of the transformation cell mixture.

About one million primary clones were divided into 20 pools (50,000 clones each pool) and each pool was plated on 245mm x 245 mm square plate containing MR2001 medium (MacConnel Research, San Diego, CA) and about 60 ug/ml carbonocillin. After incubation overnight at 37C, the colonies were scraped off the plate in about 20 ml SOC (SOC contains about 2 percent

Bactotryptone, 0.5 percent yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, and 10 mM magnesium sulfate) and were pelleted by centrifugation at about 6000 rpm for about 10 minutes. The plasmids were then recovered from the cells using Qiagen® maxi prep columns (Qiagen, Chatsworth, CA) according to the protocol suggested by the manufacturer.

About two hundred and fifty thousand clones (50 ug total plasmids/10 ug from each pool) were used to transform yeast strain YPH499 (ATCC accession number 90834) and an amylase-based signal trap assay was conducted as follows (see co-pending U.S.S.N. 09/026,959 filed 20 February 1998 for a detailed description of this technique). Around 1000 transformants were plated on a single starch-containing selection plate (15 cm diameter with a medium containing about 0.6 percent yeast nitrogen base, 2 percent glucose, 0.1 percent CAA, 1.0 X trp dropout solution, 0.7 percent potato starch azure, and 1.5 percent agarose). The plates were incubated at about 30C for 4-5 days until full development of halos was observed. The colonies in the center of the halo were picked and restreaked on a fresh plate to form single colonies. The single colonies with halos were then picked and arrayed into 96 well microtiter plates containing about 100 ul of water per well, thereby generating the "yeast colony solutions".

About ten microliters of each well of each yeast colony solution was used as template to recover the cDNA fragment from that colony through PCR. Therefore, ninety-six PCR reactions were independently performed using PCR-Ready Beads® (96 well format, Amersham-Pharmacia Biotech, Pistcataway, NJ) and the following oligonucleotides according to the manufacturer's protocol:

ACTAGCTCCAGTGATCTC (SEQ ID NO:18)

CGTCATTGTTCTCGTTCC (SEQ ID NO:19)

PCR was conducted using a Perkin-Elmer 9600 thermocycler with the following cycle conditions: 94C for 10 minutes followed by 35 cycles of 94C for 30 seconds, 55C for 30 seconds and 72C for 1 minute, after which a final extension cycle of 72C for 10 minutes was conducted. Most PCR reactions contained a single PCR product. The amplified cDNA products were purified using the Qiagen® PCR purification kit (Qiagen, Chatsworth, CA). These products were sequenced on an Applied Biosystems 373A automated DNA sequencer using the following oligonucleotide primer:

GCTATACCAAGCATACAATC (SEQ ID NO:35)

Taq dye-terminator sequencing reactions (Applied Biosystems, Foster City, CA) were conducted following the manufacturer's recommended procedures.

Each PCR fragment was translated in all six possible ways to identify those fragments which (1) had a potential signal peptide in the same direction as reporter gene; (2) had a stop codon(s) upstream of the putative methionine translation start site; and (3) appeared to lack a transmembrane domain.

One clone that met these criteria, termed "ymrs2-00009-c4", was selected for further analysis. This clone contained 5' sequence, including a putative signal sequence, but was lacking 3' sequence.

To obtain the 3' sequence of this clone, a 3' RACE reaction was performed using as a template pool

number 4 from the YmHK2 cDNA library. This YmHK2 library was prepared as follows: First strand cDNA synthesis was performed using about 2 micrograms of the RNA obtained from the hippocampus of the kainate treated mice and about 1 ug of *Not I* primer-adapter having the following sequence:

GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTT (SEQ ID NO:42)

Both the first strand and second strand cDNA synthesis reactions were performed using the Superscript® plasmid system (Gibco BRL, Grand Island, NY). After second strand synthesis, the double stranded cDNA products were ligated into the double stranded adapters of SEQ ID NOs:16 and 17.

After ligation, the cDNA was digested to completion with *Not I*, and size fractionated on a 0.8 percent agarose gel. The cDNA products larger than about 800 base pairs were selected and purified from the gel using the Qiagen® gel extraction kit (Qiagen, Chatsworth, CA). The purified cDNA products were directly ligated into *Sal I* and *Not I* digested pSport® vector (Gibco BRL, Grand Island, NY).

The ligated cDNA products were then introduced into electrocompetent *E. coli* cells called ElectroMax® DH10B (Gibco BRL, Grand Island, NY). The library was then titered.

About twelve million primary clones were obtained, and expanded into about 250 ml of LB containing about 100 ug/ml ampicillin. After overnight incubation at 37C, the plasmids were recovered using the Qiagen® maxi-prep kit (Qiagen, Chatsworth, CA).

About 20 ng of the plasmid library were used to transform the ElectroMax® DH10B electrocompetent *E. coli* cells using standard electroporation techniques. About two million transformants were divided into 40 pools (containing approximately 50,000 plasmids/pool). Each pool was then expanded into about 3 ml of LB medium containing about 100 ug/ml ampicillin. After overnight incubation at 37C, the plasmids were recovered using the Qiagen® mini-prep kit. The DNA from each pool were then stored at about minus 20C for future use.

The 3' RACE reaction was performed using about 1.5 ng of pool #4 of the YmHK2 library as a template, and using the Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) with the following oligonucleotides:

CCAGCTGCTCTGTGGCAGCCCAG (SEQ ID NO:20)

CCCAGTCACGACGTTGTAAAACGACGGCC (SEQ ID NO:21)

The reaction was conducted in a standard thermocycler (Perkin-Elmer 9600) for thirty five cycles under the following conditions: 94 C for 1 minute; 94 C for 5 seconds, and 72 C for 5 minutes. This was followed by a final extension at 72C for 10 minutes. About one microliter of the reaction products was diluted to 50 ul using TE buffer (10 mM TRIS pH 8.0 and 1 mM EDTA).

To enrich the RACE reaction for the gene of interest, a nested PCR reaction was conducted using about five microliters of the TE solution (containing the RACE reaction products as described in the preceding paragraph) together with the following oligonucleotides:

AACATGCAGCGGCTCGGGGG (SEQ ID NO:22)

GGTGACACTATAGAAGAGCTATGACGTCGC (SEQ ID NO:23)

The nested PCR reaction was incubated in a thermocycler (Perkin-Elmer 9600) using the following protocol: 94C for one minute; five cycles of 94C for 5 seconds followed by 72C for 5 minutes; five cycles of 94C for five seconds, followed by 70C for 5 minutes; and 20-25 cycles of 94C for 5 seconds followed by 68C for 5 minutes. After this PCR, the 3' RACE products and the nested PCR products were analyzed using standard agarose gel electrophoresis.

A PCR product of about 3.3 kb was identified from the nested PCR reaction. This fragment was purified using Qiagen® Gel Extraction Kit (Qiagen, Chatsworth, CA) and ligated into the vector pCRII-TOPO (Invitrogen, Carlsbad, CA) according to the procedures recommended by the manufacturer. After ligation, the products were transformed into One Shot® *E. coli* cells (Invitrogen, Carlsbad, CA) and plated on a LB (Luria broth) plate containing about 100 ug/ml ampicillin and about 1.6 mg X-gal. After overnight incubation at 37C, 12 white colonies and one blue colony were selected, and screened using PCR-Ready Beads® (Amersham-Pharmacia Biotech, Pistcataway, NJ) according to the manufacturer's recommended protocol using oligonucleotide SEQ ID NO:20 together with the following primer:

GTGCTGAGTGTCTTCCATCAGC (SEQ ID NO:24)

Two colonies were picked that had yielded PCR products of the expected size of about 192 base pairs.

These colonies were inoculated into about 3 ml of LB medium containing about 100 ug/ml ampicillin, and were incubated at 37C. The cultures were placed on a shaker for about 16 hours, and the plasmids were recovered using Qiagen® mini prep columns (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Plasmid DNA was then sequenced as described above.

A contiguous stretch of DNA of about 3366 nucleotides was assembled by combining the sequence of clone ymrs2-00009-c4 (containing 5' sequence) together with the nested PCR fragment of 3.3 kb containing 3' sequence. Within this contiguous sequence is an open reading frame of 349 amino acids. The nucleotide sequence of this novel mouse gene, referred to as DKR-3, is set forth in Figure 1. The putative amino acid sequence, as translated from the DNA sequence, is set forth in Figure 8

A BLAST search of the Genbank database using the amino acid sequence of DKR-3 revealed that this open reading frame has homology to a gene known as human rig-like 7-1 mRNA (Genbank accession number AF034208; see also Ligon *et al., J NeuroVirology*, 4:217-226 [1998]). DKR-3 also has homology to the gene for chicken lens fiber protein clfest4 (Genbank accession number D26311); the overall identity to this protein is about 50 percent with the highest homology in the middle of the protein.

Example 2: Cloning of the Human DKR-3 Gene

Mouse DKR-3 DNA can be used to search a public EST database for human homologs, resulting in the identification of the following Genbank accession numbers:

AA628979
AA349552
AA633061
AA351624
W61032
T30923
AA683017
AA324686
T08793
T31076
R14945
AA226979
W45085
AA424460
R58671
R57834
AF034208

These EST sequences were analyzed and assembled to create a putative sequence for human DKR-3. Based on this putative sequence, two oligonulceotides were designed for use in PCR in an attempt to clone the human DKR-3 gene. The sequence of these oligonucleotides is:

GAGATGCAGCGGCTTGGGGCCACCC (SEQ ID NO:25)

GCCTGGTCAGCCCACGCCTAAAG (SEQ ID NO:26)

PCR was performed using the Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) together with human fetal brain Quick-Clone® cDNA (Clontech). PCR was conducted in a thermocycler (Perkin-Elmer 9600) under the following cycle conditions: 94C for 2 minute; 94C for 30 seconds, and 72C for 2 minutes. Thirty-five

cycles were conducted after which samples were treated at 72C for 10 minutes. A single fragment of about 1150 base pairs was visible when the PCR products were visualized on a 1 percent agarose gel. This fragment was purified using the Qiagen® Gel Extraction Kit (Qiagen, Chatsworth, CA) and ligated into the vector pCRII-TOPO (Invitrogen, Carlsbad, CA). After ligation, the products were transformed into One Shoot *E. coli*® (Invitrogen, Carlsbad, CA) and plated on a LB plate containing about 100 ug/ml ampicillin and about 1.6 mg X-gal. After overnight incubation at 37C, 2 white colonies were picked and inoculated into about 3 ml of LB medium containing about 100 ug/ml ampicillin. The cultures were kept on a shaker at about 37C for about 16 hours. The plasmids were isolated using Qiagen® mini-prep columns (Qiagen, Chatsworth, CA) according to the manufacturer's recommended protocol, and the inserts were then sequenced using methods described above.

The cloned fragment is 1141 bp in length and contains an open reading frame of 350 amino acids. The nucleotide sequence is set forth in Figure 2, and the putative amino acid sequence, as translated from the DNA sequence, is set forth in Figure 9. This amino acid sequence is about 80 percent identical to the mouse DKR-3 gene. In addition, human DKR-3 is identical to the human rig-like protein fragment described by Lignon *et al., supra* between amino acids 157 and 308 of DKR-3. Significantly, the rig-like protein has an amino terminal start corresponding to amino acid 156 of DKR-3. Rig-like does not appear to be a secreted protein, and the carboxy terminal region of rig-like protein has no homology to human DKR-3. Just as for mouse DKR-3, human DKR-3 is about 54 percent identical to the chicken lens fiber protein

clfest. Human DKR-3 appears to be secreted, with a signal peptide cleavage site after either amino acid 20 or 21. Other potential cleavage sites (due to signal peptides or to other endogenous processing sites are after amino acid 16, 22, 32, and/or 41). There appear to be N-linked glycosylation sites at amino acids 96, 106, 121, and 204, which would render them preferable sites for generating substitution mutants. Human DKR-3 and mouse DKR-3 amino acid sequences differ at amino acid positions 6, 7, 11, 24, 27, 29, 30, 32, 33, 39, 81, 89, 93, 99, 101, 103, 109, 113, 115, 123, 126, 142, 156, 157, 162, 165, 173, 175, 191, 197, 198, 201, 203, 245, 247, 259, 283, 287, 292, 294, 295, 296, 298, 299, 304, 310, 311, 312, 314, 315, 329, 330, 334, 335, 336, 339, 340, 341, 342, 343, 345, and 347 (all with respect to the human DKR-3 sequence), which renders these positions preferable for generating human DKR-3 substitution or deletion variants. Based on computer analysis of the amino acid sequence of DKR-3, significant regions of the molecule include the span from about amino acids 21-145 (a potential alpha-helical region and region of potential N-linked glycosylation) such as for example amino acids 21-145, 40-145, 40-150, 45-145, and 45-150, the span from about amino acids 145-350, such as, for example 145-290, 145-300, and 145-350, and the span from about amino acids 300-350 (a second potential alpha-helical region), such as for example amino acids 310-350. Such regions would be suitable fragments of full length DKR-3.

Northern blot analysis was conducted to assess the tissue specific expression of human DKR-3. A probe for use in Northern blot analysis was prepared by PCR of human fetal brain Quick-Clone® cDNA (Clontech, Palo Alto, CA) using the following oligonucleotides:

CCTGCTGCTGGCGGCGGCGGTCCCCACGGC (SEQ ID NO:27)

GCCTGGTCAGCCCACGCCTAAAG (SEQ ID NO:28)

The PCR reaction was conducted in a thermocycler (Perkin-Elmer 9600). PCR conditions were: 94C for 2 minute; 94C for 30 seconds, and 72C for 2 and 1/2 minutes. Thirty-five cycles were conducted followed by a final extension treatment at 72C for 10 minutes. PCR products were run on a one percent agarose gel, and a band of about 1100 bp was gel purified using the Qiagen gel extraction kit (Qiagen®, Chatsworth, CA), cloned into the vector CRII-TOPO (Invitrogen, Carlsbad, CA) and sequenced to confirm that the band contained the human DKR-3 open reading frame minus the amino terminal 10 amino acids.

About twenty-five nanograms of this probe was denatured by heating to about 100C for about 5 minutes, followed by placing on ice, and then radioactively labeled with alpha-32P-dCTP using the Rediprime® labeling kit (Amersham, Arlington Heights, IL) and following the manufacturer's instructions. A human multiple tissue Northern blot was purchased (Clontech, Palo Alto, CA) and was first prehybridized in about 5 ml of Clontech Express® hybridization buffer at about 68C for 30-60 minutes. After prehybridization, the labeled probe was added to the solution and allowed to hybridize for about 60 minutes. After hybridization, the blot was first washed with 2xSSC plus 0.05 percent SDS at room temperature for about 30 minutes, then washed with 0.1xSSC plus 0.1 percent SDS at about 65C for about 30 minutes. The blot was dried briefly and then exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). After overnight exposure,

the image of the blot was analyzed on a Storm 820 machine (Molecular Dynamics, Sunnyvale, CA) with Imagequat software (Molecular Dynamics, Sunnyvale, CA).

The size of the human DKR-3 RNA transcript is about 2.6 kb. The results of the Northern blot analysis indicate that human DKR-3 is highly expressed in adult heart and brain, although weak expression in placenta, adult lung, skeletal muscle, kidney, and pancreas is also apparent. A second smaller transcript is apparent in adult pancreas, and could result from degradation of the full length transcript.

To evaluate the role of this gene in cancer, a variety of human cancer cell lines were analyzed for the presence or absence of DKR-3 RNA transcript.

The glioblastoma cell lines Hs 683; A 172; SNB-19; U-87MG; and U-373MG are all from ATCC, and cultured in the media recommended by ATCC.

Normal human mammary epithelial cells (NMECs) derived from reduction mammoplasties were purchased from Clonetics Corp. (San Diego, CA) and the Corriel Institute (Camden, N.J.). The immortalized breast epithelial cell line MCF-10 and the ER+ cell line MCF-7 can be obtained from the American Type Culture Collection. The ER+ BT20T cells were provided by Dr. K. Keyomarsi (N.Y. State Dept. of Health). Immortalized 184A1 and other breast cancer cells including T47-D, ZR75-1, and BT474, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB- 453, MD-MBA-468, HS578T and SKBr3 were all obtained from the American Type Culture Collection (10801 University Blvd., Manassas, VA).

NMECs, 184A1 and MCF10 cells were cultured in a modified DME/F12 medium (Gibco/BRL, Grand Island, NY) supplemented with 10 mM Hepes, 2mM glutamine, 0.1 mM nonessential amino acids, 0.5 mM ethanolamine, 5 mg/ml transferrin, 1 mg/ ml Bovine serum albumin, 5.0 ng/ml

sodium selenite, 20 ng/ml triiodothyronine, 10 ng/ml EGF, 5 μg/ml insulin and 0.5 μg/ml hydrocortisone (DMEM/F12C) (Ethier *et al, Cancer Letters*, 74:189-195 [1993]). The ER+ and ER+ breast cancer cells were cultured in Alpha or Richter improved minimal essential medium (MEM) (Gibco/BRL) supplemented with 10 mM Hepes, 2mM glutamine, 0.1 mM nonessential amino acids, 10 percent fetal bovine serum and 1 μg/ml insulin.

Normal human bronchial and cervical epithelial cells were purchased from Clonetics Corp. (San Diego, CA). Normal cervical epithelial cells were culture in KBM2 (Clonetics Corp. San Diego, CA) supplemented with 13 mg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 2 ng/ml EGF, 0.5 mg/ml epinephrine, 0.1 ng/ml retinoic acid, 5 μg/ml transferrin, 6.5 ng/ml triiodothyronine and 5 μg/ml insulin. Normal bronchial epithelial cells were cultured in BEBM (Clonetics Crop., San Diego, CA) supplemented with 0.5 mg/ ml hydrocortisone, 0.5 ng/ ml EGF, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ ml insulin, 0.1 ng/ml retinoic acid and 5.5 ng/ ml triiodthyronine.

The lung cancer cell lines H1299, H23, H358, H441, H460, H520, H522, H727, H146, H209, H446, H510A, H526, and H889 and the cervical cancer cells Caski, C-4-I, MS751, SiHa and C-33-A were all obtained from the American Type Culture Collection. The lung cancer cells were cultured in RPMI (MEM) (Gibco/BRL) supplemented with 10 mM Hepes, 2 mM glutamine, 0.1 mM nonessential amino acids and 10 percent fetal bovine serum (FBS). The cervical cancer cells were cultured in Earles MEM supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate and 10 percent FBS. All cells were routinely screened for mycoplasma

contamination and maintained at about 37°C in an atmosphere of about 6.5 percent CO_2.

Total RNA was prepared by lysing cell monolayers in guanidinium isothiocyanate and centrifuging over a 5.7 M CsCl cushion as described previously (Gudas, *Proc. Natl. Acad. Sci USA*, 85:4705-4709 [1988]). RNA (about 20 ug) was electrophoresed on denaturing formaldehyde gels, transferred to MagnaNT membranes (Micron Separations Inc., Westboro, MA) and cross-linked with UV irradiation.

The blots were prehybridized, probed, and washed under the same conditions as those set forth above for the tissue blot. The blots were dried briefly and then exposed to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). After overnight exposure, the image of the blot was analyzed on a Storm 820 machine with Imagequat software (both from Molecular Dynamics).

The results are shown in Figures 15A-15D. As can be seen in Figure 15A, expression of DKR-3 is decreased in most of the breast cancer cell lines as compared to the normal cell lines. Figure 15B indicates that DKR-3 expression is decreased in the non-small cell lung cancer cell lines, and in most of the small cell lung cancer cell lines as well. Figure 15C indicates that expression of DKR-3 is decreased in three glioblastoma cell lines (SNB-19, U-87MG, and U-373MG) that are capable of forming tumors in nude mice (the other two cell lines, Hs 683 and A 172 do not form tumors in nude mice). Figure 15D indicates that expression of DKR-3 is reduced in cervical cancer cell lines as compared to normal and immortalized cells.

Example 3: Cloning of the Human DKR-1 Gene

Human and mouse DKR-3 cDNA and amino acid sequences were used to search Genbank using the BLAST program in an attempt to identify DKR-3 related genes. A number of EST (expressed sequence tags) were found and were analyzed to determine whether the sequences overlapped. Using the following human EST accessions, a novel gene, termed DKR-1, was predicted.

AA336797
R27865
W39690
AA043027
HUM517H04B
AA143670
W51876
N94525
AA641247
AA137219
AA115249
AA031969
AA136192
AA032060
AA035583
AA207078
AA371363
AA037322
AA088618
W46873
AA115337
AA693679
W30750
H83554

PCR was conducted in an attempt to clone the full length gene, and the following two oligonucleotides were used for PCR:

CCCGGACCCTGACTCTGCAGCCG (SEQ ID NO:29)

GAGGAAAAATAGGCAGTGCAGCACC (SEQ ID NO:30)

PCR was performed using the Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) containing the oligonucleotides listed above and human placenta Quick-Clone® cDNA (Clontech, Palo Alto, CA). The reaction was conducted according to the manufacturer's recommendations. Thirty-five cycles of PCR were conducted in a thermocycler (Perkin-Elmer 9600) under the following conditions: 94C for 2 minutes; 94C for 30 seconds, and 72C for 1-1/2 minutes, followed by a final extension at 72C for 10 minutes.

After cycling, the PCR products were analyzed on a one percent agarose gel. A single band of about 1200 base pairs in length was detected after agarose gel electrophoresis. This fragment was purified using the Qiagen® gel extraction kit (Qiagen, Chatsworth, CA) and ligated into the vector pCRII-TOPO (Invitrogen, Carlsbad, CA) using standard ligation procedures. After ligation, the products were transformed into One Shoot® competent *E. coli* cells according to the procedures recommended by manufacturer (Invitrogen, Carlsbad, CA). The transformed *E. coli* cells were plated on a LB plate containing about 100 ug/ml ampicillin and about 1.6 mg X-gal.

After overnight incubation at about 37C, two white colonies were picked and inoculated into about 3 ml of TB containing 100 ug/ml ampicillin. The culture

was incubated at about 37C for about 16 hours, plasmids were then recovered using Qiagen® mini-prep columns (Qiagen, Chatsworth, CA) and sequenced. Both colonies contained the same insert.

The insert is 1193 base pairs, and is referred to as human DKR-1. The sequence of this gene is set forth in Figure 3. This gene contains an open reading frame of 266 amino acids. The amino acid sequence is set forth in Figure 10. A stop codon is present upstream of the first methionine, indicating the first methionine is likely to be the amino terminus of the protein. Human DKR-1 has a predicted signal peptide with a predicted signal peptide cleavage site between amino acids 19 and 20.

The gene has about 80 percent homology to the mouse gene *dkk-1* (Glinka *et al., supra*), however the mouse *dkk-1* gene is 272 amino acids in length while human DKR-1 is 266 amino acids in length. Human DKR-1 differs from mouse *dkk-1* at amino acid positions 3, 4, 5, 7, 8, 10, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 29, 53, 55, 62, 66, 69, 77, 93, 98, 101, 105, 106, 123, 139, 140, 143, 144, 153, 155, 157, 158, 163, 164, 165, 169, 175, 178, 197, 224, and 244. In addition, the alignment of human DKR-1 and mouse *dkk*-1 shows one gap in human DKR-1 between amino acids 37 and 38, and two gaps between 103 and 104, 146 and 147, and 165 and 166. Glinka *et al*. state on page 362 of their article that "Coordinates of Xenopus dkk family members have been deposited in Genbank with the following accession numbers...hdkk-1 AA207078." However, forward three frame translations of AA207078 by the inventors herein showed no homology to the published mouse and *Xenopus* dkk-1 sequences, or to the human DKR-1 sequence, except in the 3' end of this accession, which exhibits a 95 percent identity to human DKR-1 from amino acids 81-

179, indicating that AA207078 does not encode full length human *dkk-1*. Significantly, AA207078 is missing amino acids 1-90 and 180-350 of human DKR-1 which includes the signal peptide and the second cysteine right domain respectively.

Example 4: Cloning of the Mouse DKR-2 Gene

Genbank accession number AA265561 (a mouse sequence) has homology to both human DKR-1 and human DKR-3 at the amino acid level based primarily on its cysteine pattern.

To extend this EST sequence in both the 5' and 3' directions, the following oligonucleotides were designed:

GCCACAGTCCCCACCAAGGATCATC (SEQ ID NO:31)

GATGATCCTTGGTGGGGACTGTGGC (SEQ ID NO:32)

CTGCAAACCAGTGCTCCATCAGGG (SEQ ID NO:33)

CCCTGATGGAGCACTGGTTTGCAG (SEQ ID NO:34)

Separately, 5' RACE and 3' RACE reactions were performed according to the manufacturer's protocol using mouse heart Marathon-Ready® cDNA and the Advantage® cDNA PCR kit (both from Clontech, Palo Alto, CA) and using oligonucleotide SEQ ID NOs: 31 and 34. The RACE reactions were incubated in a thermocycler (Perkin-Elmer 9600) using the following cycling conditions: 94C for one minute; five cycles of 94C for 5 seconds followed by 72C for 5 minutes; five cycles of 94C for five seconds, followed by 70C for 5

minutes; and 20-25 cycles of 94C for 5 seconds followed by 68C for 5 minutes.

To enrich each RACE reaction for the desired product, about one microliter of each of the RACE PCR products was added together, and the mixture was diluted to about 50 ul using TE buffer. About five microliters of this solution were used to conduct nested PCR reactions. The Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) and oligonucleotide SEQ ID NOs: 32 and 33 were used for the 5' and 3' nesting reactions, respectively. The nested PCR reactions were incubated in a thermocycler (Perkin-Elmer 9600) using the following program for thirty five cycles: 94C for 1 minute; 94C for 5 seconds; and 72C for 2 minutes. A final extension was then conducted at 72C for 10 minutes. The PCR products were analyzed using a one percent agarose gel.

Several fragments ranging from about 500 bp to about 1500 base pairs were obtained from the 5' nested PCR reaction, and two fragments of about 1900 bp and 450 bp were obtained from the 3' nested PCR reaction. These PCR products were purified using the Qiagen® PCR purification kit (Qiagen, Chatsworth, CA) and were then ligated into the vector pCRII-TOPO (Invitrogen). The ligation products were transformed into OneShot® *E. coli* cells (Invitrogen, Carlsbad, CA), and the cells were then plated on to two X-gal containing plates (one for each reaction)as described above.

Eight white colonies from each plate were picked and PCR selected via RACE reactions using the Clontech primer AP2 and the oligonucleotide SEQ ID NO:32 (for the 5' RACE) or the oligonucleotide SEQ ID NO:33 (for the 3' RACE). Three colonies from each plate that contained the correct size fragments were

cultured, and the plasmids were isolated and sequenced using procedures described above.

Three clones, 9813302, 9813304 and 9813305 contained sequence which extended the EST sequence in the 5' direction. One clone, 9813308, contained sequence which extended the EST sequence in the 3' direction. A continuous sequence of 2678 base pairs was thus assembled using the sequence of clones 9813308, 9813304, and the EST AA265561. This full length DNA has been termed DKR-2, and the sequence is set forth in Figure 4. The corresponding amino acid sequence is set forth in Figure 11.

Within the amino acid sequence is an open reading frame of 259 amino acids. This protein has approximately 38 percent identity with mouse *dkk*-1 at the amino acid level. Mouse DKR-2 has a predicted signal peptide with a signal peptide cleavage site between amino acids 33 and 34.

Example 5: Cloning of the Human DKR-2 Gene

The Genbank EST database was searched using the BLAST program with both DNA and amino acid sequences from human DKR-1 and human DKR-3, and one human EST, W55979, was identified that showed homology to both human DKR-1 and human DKR-3 at the amino acid level based on its cysteine pattern. W55979 is about 88 percent identical to mouse DKR-2 at the DNA level, and about 93 percent identical to mouse DKR-2 at the amino acid level.

A BLAST search of Genbank W55979 indicated that W55979 has homology to BAC clone number B284B3 (Genbank accession number AC003099). BAC clone B284B3 is 95129 base pairs in length. Three portions of W55979 are homologous to three different regions of BAC

clone B284B3, indicating that human DKR-2 has at least three exons. A 3' sequence of 556 bp in length was assembled based on the sequences of both BAC clone B284B3 and W55979, and it was determined that this sequence is the 3' portion of the human ortholog of mouse DKR-2. Within this 3' sequence of human DKR-2 is an open reading frame of 174 amino acids, and a stop codon is present after amino acid 174. This 3' sequence of human DKR-2 is about 97 percent identical to mouse DKR-2.

To obtain the 5' end sequence of human DKR-2, a 5' RACE reaction was performed using Clontech human heart Marathon-Ready® cDNA and the Advantage® cDNA PCR kit, together with oligonucleotide SEQ ID NO:34. The RACE reaction was performed according to the manufacturer's protocol. The 5' RACE reaction products were then subjected to nesting PCR to enrich for the 5' sequence using the Advantage® cDNA PCR kit and oligonucleotide SEQ ID NO:32. The PCR conditions for both the 5' RACE reaction and the nested PCR reaction were the same as those described in Example 4.

The nested PCR products were purified using the Qiagen® (Qiagen, Chatsworth, CA) PCR purification kit, and were ligated into the vector Zero-Blunt® (Invitrogen, San Diego, CA) according to the procedures recommended by the manufacturer. The ligation products were transformed into OneShot® *E. coli* cells which were then plated on X-gal containing plates as described above.

After overnight culturing, three white colonies were picked and were inoculated into about 3 ml of TB containing about 100 ug/ml ampicillin. The cultures were allowed to grow for about 16 hours, after which the plasmids were isolated using Qiagen® mini-

prep columns (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. The sequence of each insert was then obtained.

One of the 5'-RACE clones, termed 9812826, extended the human DKR-2 sequence 5'-terminally. A contiguous sequence of 1531 bp in length was assembled using this clone 9812826 together with the human DKR-2 3'sequence. Within this contiguous sequence is an open reading frame of 259 amino acids. The human DKR-2 gene has a predicted signal peptide of about 33 amino acids, with a predicted cut site between amino acids 33 and 34, and is about 95 percent identical to mouse DKR-2 at the amino acid level. The amino acid positions that differ between human and mouse DKR-2 include (with respect to the numbering of the human sequence) 7, 12, 28, 48, 50, 58, 71, 102, 119, 170, 173, and 191, rendering these positions preferable for generating amino acid substitution or deletion variants.

An alternative spliced isoform of human DKR-2 was discovered when PCR was conducted using human heart Marathon-Ready® cDNA (Clontech, Palo Alto, CA) and the Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) together with the following oligonucleotides:

GGGTTGAGGGAACACAATCTGCAAG (SEQ ID NO:36)

GTCTGCAATTGATGATGTTCCTCAATGG (SEQ ID NO:37)

PCR was conducted using parameters set forth in the manufacturer's protocol. PCR products were analyzed by agarose gel electrophoresis, and two PCR products were obtained. The bands corresponding to these products were gel purified as described above, amplified and purified as described above, and then sequenced. One product corresponded to full length

DKR-2, however, the other band corresponded to an isoform of DKR-2. This isoform has an open reading frame of 207 amino acids, and appears to be missing an exon. This isoform is referred to as human DKR-2a. The DNA sequence of human DKR-2a is set forth in Figure 6, and the amino acid sequence as translated from the DNA is set forth in Figure 13.

Example 6: Cloning of the Human DKR-4 Gene

A human EST that showed significant homology to human DKR-1 and human DKR-3 on protein level was identified in Genbank. This sequence, Genbank accession number AA565546, has a cysteine pattern that is similar to that of human DKR-1 and human DKR-3.

A BLAST search of Genbank showed no human ESTs overlapping with AA565546. Therefore, to extend the EST sequence in the 5' direction, a 5' RACE reaction was performed using human heart Marathon-Ready® cDNA (Clontech, Palo Alto, CA) together with the Advantage® cDNA PCR kit (Clontech, Palo Alto, CA) and the following oligonucleotide:

CCAGGGCCACAGTCGCAACGCTGG (SEQ ID NO:38)

The RACE reaction was performed according to the protocol provided with the Advantage® kit. After 5' RACE, the products were nested to enrich for the desired 5' sequence using the Advantage® cDNA PCR kit according to the manufacturer's recommendations, together with the following oligonucleotide:

CTCCCTCTTGTCCCTTCCTGCCTTG (SEQ ID NO:39)

After the nested PCR reaction, the products were purified using the Qiagen® PCR purification kit (Qiagen, Chatsworth, CA), ligated into the vector pCRII-TOPO (Invitrogen, Carlsbad, CA), and transformed into OneShot® *E. coli* cells as described above. After transformation, the cells were plated on a LB plate containing about 100 ug/ml ampicillin and about 1.6 mg X-gal.

After overnight incubation at 37C, four white colonies were picked from the plate and were inoculated in about 3 ml TB containing about 100 ug/ml ampicillin. The cultures were incubated at about 37C for about 16 hours. The plasmids were then recovered using Qiagen® mini-prep columns (Qiagen, Chatsworth, CA) and sequenced.

Two clones, termed 9813563 and 9853564, were found to contain the 5' sequence of human DKR-4.

To obtain the 3' sequence of human DKR-4, a 3' RACE reaction was performed using human uterus Marathon-Ready® cDNA (Clontech, Palo Alto, CA) together with the Advantage® cDNA PCR kit (Clontech) and the following oligonucleotide:

CAAGGCAGGAAGGGACAAGAGGGAG (SEQ ID NO:40)

The 3' RACE reaction was performed according to the manufacturer's recommendations. After the RACE reaction, the products were nested using the Advantage® cDNA PCR kit and the following oligonucleotide:

CCAGCGTTGCGACTGTGGCCCTGG (SEQ ID NO:41)

The parameters for PCR were 94C for 1 minute followed by thirty five cycles of 94C for 5 seconds and then 72C for 2 minutes, after which a final extension of 70C for 10 minutes was conducted. After the nesting reaction, the products were analyzed on a 1 percent agarose gel. A single band of about 1200 bp in length was observed. This band was purified from the gel using methods described above, and was then cloned into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced. Sequence of this band indicated that it contained the 3' sequence of human DKR-4., and this sequence was assembled together with the 5' sequence (from clones 9813563 and 9853564) to generated the full length sequence of human DKR-4. This sequence is set forth in Figures 7 (DNA sequence) and 14 (translated amino acid sequence). The polypeptide is 224 amino acids in length and is about 34 percent identical to human DKR-1 at the amino acid sequence level.

Example 7: Expression of Human DKR-1 in Bacteria

PCR amplification employing the primer pairs and template described below were used to generate a recombinant form of human DKR-1. One primer of each pair introduces a TAA stop codon and a unique *BamHI* site following the carboxy terminus of the gene. The other primer of each pair introduces a unique *NdeI* site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling was performed using standard recombinant DNA methodology. The PCR products were purified, restriction digested, and inserted into the unique *NdeI* and *BamHI* sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic *E. coli* host GM121 (deposited with the American Type Culture

Collection on XX as accession number XX). Other commonly used *E. coli* expression vectors and host cells are also suitable for expression by one skilled in the art. After transformation, positive clones were selected and examined for expression of the recombinant gene product.

The construct pAMG21-human DKR-1-24-266 was engineered to be 244 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-His-Pro-Leu-Leu-Gly (SEQ ID NO:43)

Thr-Cys-Gln-Arg-His (SEQ ID NO:44)

The template used for PCR was human DKR-1 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct:

GTTCTCCTCATATGCATCCATTATTAGGCGTAAGTGCCACCTTGAACTCGGTTCT CAAT (SEQ ID NO:45)

TACGCACTGGATCCTTAGTGTCTCTGACAAGTGTGAAG (SEQ ID NO:46)

Transformed *E. coli* strain GM121 containing pAMG21-human DKR-1-24-266 were grown in 2X YT media containing 20 micrograms/ml kanamycin at 30C until the culture reached an optical density of about 600 nm of about 0.5. Induction of DKR-1 protein expression was achieved by addition of *Vibrio fischeri* synthetic autoinducer to 100 ng/ml final and incubation of the culture at either 30 °C or 37 °C for about 9 hours further with shaking. In addition, as a uninduced control, for each culture no autoinducer was added to an aliquot of the culture, but the culture was also

incubated for about 9 hours further at about 30C with shaking along with the induced cultures. After about 9 hours, the optical density of cultures were measured at 600 nm, an aliquot of cultures were examined by oil emersion microscopy at 1600X magnification, and aliquots of cultures were pelleted by centrifugation. Bacterial pellets of cultures were processed for SDS-polyacrylamide gel electrophoresis on a 14 percent gelto examine levels of protein produced in crude lysates and for N-terminal sequencing confirmation of the recombinant gene product. The gel was stained with Coomassie blue.

The results are shown in the photo of Figure 16. Lane 1 contains molecular weight markers; Lanes 2 and 5 contain crude lysates of uninduced control cells incubated at 30C; Lanes 3 and 6 are crude lysates of induced cells cultured at 30C; Lanes 4 and 7 are crude lysates of induced cells cultured at 37C. The arrow on the left of Lane 1 indicates the expected location of human DKR-1-24-266. As can be seen, large amounts of recombinant protein were observed in crude lysates of induced cultures at both 30 °C and 37 °C (Lanes 3 and 6, and 4 and 7). Microscopic analysis of bacterial cells revealed most cells contained at least one inclusion body, suggesting that at least some of the protein may be produced in the insoluble fraction of *E. coli.*

<u>Example 8: Expression of DKR-2 in Bacteria</u>

PCR amplification employing the primer pairs and templates described below were used to generate various forms of DKR-2. One primer of each pair introduces a TAA stop codon and a unique *BamHI* site following the carboxy terminus of the gene. The other primer of each pair introduces a unique *NdeI* site, a N-

terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling was performed using standard recombinant DNA methodology. The PCR products were purified, restriction digested, and inserted into the unique *NdeI* and *BamHI* sites of vector pAMG21 (ATCC accession no. 98113) and transformed into either prototrophic *E. coli* host GM121 or GM94 (GM 94 was deposited with the ATCC on XX as accession number XX). Other commonly used *E. coli* expression vectors and host cells are also suitable for expression. After transformation, positive clones were selected and examined for expression of the recombinant gene product.

The construct pAMG21-human DKR-2-26-259 was engineered to be 235 amino acids in length and have the following N-terminal and the following C-terminal amino acids, respectively:

Met-Ser-Gln-Ile-Gly-Ser (SEQ ID NO:47)

Val-Cys-Gln-Lys-Ile (SEQ ID NO:48).

The template used for PCR was human DKR-2 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct.

GTTCTCCTCATATGTCTCAAATTGGTAGTTCTCGTGCCAAACTCAACTCCATCAA G (SEQ ID NO:49)

TACGCACTGGATCCTTAAATTTTCTGACACACATGGAGT (SEQ ID NO:50)

The construct pAMG21 mouse DKR-2-26-259 was engineered to be 235 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-Ser-Gln-Leu-Gly-Ser (SEQ ID NO:51)

Val-Cys-Gln-Lys-Ile (SEQ ID NO:52)

The template used for PCR was mouse DKR-2 cDNA, and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct.

GTTCTCCTCATATGTCTCAATTAGGTAGCTCTCGTGCTAAACTCAACTCCATCAA GTCC (SEQ ID NO:53)

TACGCACTGGATCCTTAGATCTTCTGGCATACATGGAGT (SEQ ID NO:54)

Transformed *E. coli* GM121 or GM94 containing either pAMG21-human DKR-2-26-259 or pAMG21-mouse DKR-2-26-259 plasmid were grown in 2X YT media containing 20 μg/ml kanamycin at 30 °C until the culture reached an optical density at 600 nm of about 0.5. Induction of DKR-2 protein expression was achieved by addition of *Vibrio fischeri* synthetic autoinducer to 100 ng/ml final and incubation of the culture at either 30C or 37C for about 5 or 9 hours further with shaking. In addition, as a uninduced control, for each culture no autoinducer was added to an aliquot of the culture, but the culture was also incubated for about 5 or 9 hours further at 30C with shaking along with the induced cultures. After either 5 or 9 hours incubation, the optical density of cultures were measured at about 600nm, an aliquot of cultures were examined by oil emersion microscopy at 1600X magnification, and aliquots of cultures were pelleted by centrifugation. Bacterial pellets of cultures were processed for SDS-polyacrylamide gel electrophoresis on a 14 percent gel to examine levels of protein produced

in crude lysates and for N-terminal sequencing confirmation of the recombinant gene product. The gel was stained with Coomassie blue.

The results are shown in Figure 16, Lanes 8-10 (human DKR-2 polypeptide)and in Figure 17 (mouse DKR-2 polypeptide). In Figure 16, Lane 8 contains crude lysate of uninduced control cells; Lane 9 contains crude lysate of induced cells cultured at 30C, and Lane 10 contains crude lysate of induced cells cultured at 37C. The arrow to the left of Lane 10 indicates the expected location of human DKR-2-26-259. As can be seen, significant amounts of polypeptide were generated in the induced cultures whether grown at 30C or 37C, while the uninduced cells did not produce a large amount of polypeptide. Figure 17 shows the results of polypeptide production of mouse DKR-2-26-259. Lane 1 is molecular weight markers. Lanes 2-4 are one clone of *E coli* cells transfected with the DKR-2 plasmid, while Lanes 5-7 are a second clone transfected with the same plasmid. Lanes 2 and 5 are crude lysates of uninduced control cells; Lanes 3 and 6 are crude lysates of induced cells cultured at 30C; and Lanes 4 and 7 are crude lysates of cells cultured at 37C. The arrows to the left of Lanes 4 and 7 indicate the expected location of the DKR-2 polypeptide. As can be seen, large amounts of recombinant protein were observed in crude lysates of induced cultures at 37C but not at 30C. Microscopic analysis of bacterial cells revealed most cells contained at least one inclusion body, suggesting that at least some of the protein may be produced in the insoluble fraction of *E. coli*.

Example 9: Expression of DKR-3 in Bacteria

PCR amplification employing the primer pairs and templates described below were used to generate various forms of DKR-3. One primer of each pair introduces a TAA stop codon and a unique *SacII* site following the carboxy terminus of the gene. The other primer of each pair introduces a unique *NdeI* site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling was performed using standard recombinant DNA methodology. The PCR products were purified, restriction digested, and inserted into the unique NdeI and SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic *E. coli* host GM121. Other commonly used *E. coli* expression vectors and host cells are also suitable for expression by one skilled in the art. After transformation, positive clones were selected, plasmid DNA was isolated and the sequence of the DKR-3 gene insert was confirmed.

The construct pAMG21-human DKR-3-23-350 was engineered to be 329 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-Pro-Ala-Pro-Thr-Ala (SEQ ID NO:55)

Gly-Gly-Glu-Glu-Ile (SEQ ID NO:56).

The template used for PCR was human DKR-3 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct.

GTTCTCCTCATATGCCTGCTCCAACTGCAACTTCGGCTCCAGTCAAGCCCGGCC (SEQ ID NO:57)

TACGCACTCCGCGGTTAAATCTCTTCCCCTCCCAGCA (SEQ ID NO:58)

The construct pAMG21-human DKR-3-33-350 was engineered to be 319 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-Lys-Pro-Gly-Pro-Ala (SEQ ID NO:59)

Gly-Gly-Glu-Glu-Ile SEQ ID NO:60)

The template used for PCR was human DKR-3 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct:

GTTCTCCTCATATGAAACCAGGTCCAGCCTTAAGCTACCCGCAGGAGGAGGCCA (SEQ ID NO:61)

TACGCACTCCGCGGTTAAATCTCTTCCCCTCCCAGCA (SEQ ID NO:62)

The construct pAMG21-human DKR-3-42-350 was engineered to be 310 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-Gln-Glu-Glu-Ala-Thr (SEQ ID NO:63)

Gly-Gly-Glu-Glu-Ile (SEQ ID NO:64)

The template used for PCR was human DKR-3 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct:

GTTCTCCTCATATGCAAGAAGAAGCTACTCTGAATGAGATGTTCCGCGAGGTT (SEQ ID NO:65)

TACGCACTCCGCGGTTAAATCTCTTCCCCTCCCAGCA (SEQ ID NO:66)

The construct pAMG21-mouse DKR-3-33-349 was engineered to be 318 amino acids in length and have the following N-terminal and C-terminal residues, respectively:

Met-Glu-Pro-Gly-Pro-Ala (SEQ ID NO:67)

Gly-Glu-Glu-Glu-Ile (SEQ ID NO:68)

The template used for PCR was mouse DKR-3 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct:

GTTCTCCTCATATGGAACCAGGTCCAGCTTTAAACTACCCTCAGGAGGAAGCTA (SEQ ID NO:69)

TACGCACTCCGCGGTTAAATCTCCTCCTCTCCGCCTA (SEQ ID NO:70)

Transformed *E. coli* GM121 containing the various pAMG21 DKR-3 plasmids described above were grown in 2X YT media containing 20 micrograms/ml kanamycin at 30 °C until the culture reached an optical density at 600 nm of about 0.5. Induction of DKR-3 polypeptide expression was achieved by addition of *Vibrio fischeri* synthetic autoinducer to 100 ng/ml final concentration and incubation of the culture at either 30 or 37C for about 6 hours further with shaking. In addition, as a uninduced control, for each culture no autoinducer was added to an aliquot of the

culture, but the culture was also incubated for about 6 hours further at 30C with shaking along with the induced cultures. After about 6 hours, the optical density of cultures were measured at about 600 nm, an aliquot of cultures were examined by oil emersion microscopy at 1600X magnification, and aliquots of cultures were pelleted by centrifugation. Bacterial pellets of cultures were processed for SDS-polyacrylamide gel electrophoresis to examine levels of protein produced in crude lysates, or bacterial pellets were processed to determine whether the recombinant protein was in the soluble or insoluble fraction of *E. coli* and for N-terminal sequencing confirmation of the recombinant gene product. The results are shown as photos of the SDS gels in Figures 18 and 19. In Figure 18, Lane 10 is molecular weight markers, and Lanes 1-9 are crude lystes of bacterial cells. Lane 1 is crude lysate of uninduced control cells; Lanes 2, 4, 6, and 8 are crude lysates of induced cells cultured at 30C; Lanes 3, 5, 7, and 9 are induced cells cultured at 37C. Lanes 1-5 contain lysates of cells transfected with the pAMG21-human DKR-3-23-350 construct; and Lanes 6-9 contain lysates of cells transfected with the pAMG21-human DKR-3-33-350 construct. The arrows to the left of Lane 2 and the right of Lane 9 indicate the expected location of the DKR-3 polypeptides. Figure 19 contains molecular weight markers in Lane 10; Lanes 1-5 are crude lysates of cultured cells transfected with the pAMG21-human DKR-3-42-350 construct; Lanes 6-9 are crude lysates of cells transfected with the pAMG21-mouse DKR-3-33-349 construct. Lanes 1 and 6 are uninduced controls; Lanes 2, 4, 7, and 8 are crude lysates of induced cells cultured at 30C (two different clones of each construct); Lanes 3, 5, and 9 are crude lysates of induced cells cultured at 37C (two separate

clones of the human DKR-3-42-350 construct in Lanes 3 and 5). The arrow to the right of Lane 9 indicates the expected location of the mouse DKR-3 polypeptides; the arrow to the left of Lane 4 indicates the expected location of human DKR-3 polypeptide. As can be seen, all DKR-3 constructs produced large amounts of recombinant protein in *E. coli*. No inclusion bodies could be detected by oil emersion microscopy, and the recombinant polypeptides were mostly found in the soluble fraction of the cells.

Example 10: Expression of DKR-4 in Bacteria

PCR amplification employing the primer pairs and template described below were used to generate a recombinant form of human DKR-4. One primer of each pair introduces a TAA stop codon and a unique *BamHI* site following the carboxy terminus of the gene. The other primer of each pair introduces a unique *NdeI* site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling was performed using standard recombinant DNA methodology. The PCR products were purified, restriction digested, and inserted into the unique *NdeI* and *BamHI* sites of the vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic *E. coli* host GM94. Other commonly used *E. coli* expression vectors and host cells are also suitable for expression. After transformation, positive clones were selected and will be examined for expression of the recombinant gene product.

The construct pAMG21-human DKR-4-19-224 was engineered to be 207 amino acids in length and have the

following N-terminal and C-terminal residues, respectively:

Met-Leu-Val-Leu-Asp-Phe (SEQ ID NO:71)

Lys-Ile-Glu-Lys-Leu (SEQ ID NO:72)

The template used for PCR was human DKR-4 cDNA and the following oligonucleotides were the primer pair used for PCR and cloning this gene construct:

GTTCTCCTCATATGTTAGTTTTGGATTTCAACAACATCAGGAGCTCT (SEQ ID NO:73)

TACGCACTGGATCCTTACAGTTTTTCTATTTTTTGGCATACTCTTAATC (SEQ ID NO:74)

It is anticipated that DKR-4 polypeptide could be prepared using the PCR product as described above for the other DKR polypeptides.

EXAMPLE 11: Production and Purification of DKR-3 Polypeptide in Mammalian Cells

Human DKR-3 cDNA was cloned onto the mammalian expression vector pcDNA3.1(-)/mycHis (Invitrogen, Carlsbad, CA) and the vector construct was amplified using the Qiagen maxi-prep kit (Qiagen, Chatsworth, CA)standard ligation techniques.

Human embryonic kidney 293T cells (American Type Culture Collection) were cultured in 10 cm dishes, and grown to about 80 percent confluence. The cells were then transfected with the vector construct using the DMRIE-C® liposome formulation (Gibco BRL, Grand Island, NY) as follows. About 240 microliters of

DMRIE-C® were added to 8 ml of Optimem medium. About 40 ul (equivalent to about 56 micrograms) of purified vector construct was then added to another 8 ml of Optimem. After mixing and incubation at room temperature for about 15 minutes, 2 ml of this solution was added to each of 8 plates. After about 5 hours, the medium was aspirated and 10 ml of DME medium containing about 10 percent fetal calf serum was added. The cells were incubated 16-18 hours after which the medium was removed and about 10 ml of SF Optimem medium per well without phenol red were added. After about 24 hours, this medium, the "conditioned medium" was harvested, passed over a 0.22 micron filter and stored at 4°C. The cells were then incubated in another 10 ml of SF Optimem per plate. After 24 hours, this medium was collected, filtered and also stored at 4°C.

The conditioned media was added to a buffer containing 50 mM $NaPO_4$, pH8, and 250 mM sodium chloride, and passed over a column of nickel-Sephadex (Qiagen, Chatsworth, CA). Non-specifically bound proteins were eluted using the same buffer containing 10 mM imidazole, followed by the same buffer containing 20 mM imidazole. DKR-3 was then eluted using 125 mM-250 mM imidazole. Fractions from the column were subjected to 12 percent SDS gel electrophores and silver stained. The results are shown in Figure 20. Lane 2 contains material that was loaded on to the gel. Lane 3 contains the flow through fraction after loading the column with conditioned medium, Lanes 4, 5, 6, and 7 contain column fractions after treatment with 10, 20, 125, and 250 mM imidazole. Molecular weight standards are shown in Lane 8. As can be seen a single band of protein of the correct molecular weight is seen in Lanes 5 and 6, indicating that this procedure resulted in generation of purified DKR-3 protein (attached to

myc and His tags). Smearing of the protein band may be due to glycosylation. Separately, a Western blot was run to confirm that the purified protein did indeed have a His tag (indicating that the fusion protein DKR-3 mycHis had been produced). The Western blot was prepared using standard procedures and was proved with a polyclonal anti-His-HRP antibody (Invitrogen, Carlsbad, CA). A photo of the Western blot is shown in Figure 21; the Lanes correspond to that for the gel (described immediately above). As can be seen, there is antibody binding in Lanes 2, 5, and 6, indicating that DKR-3 mycHis was loaded on to the column and was eluted in the 20 and 125 mM imidazole washes.

Example 12: Anchorage Independent Growth Assay

A distinguishing feature of many cancer cell lines is their ability to grow in an anchorage independent manner. Whereas normal cells will only grow and divide until they come in contact with their neighbors, cancer cells continue to grow and divide after contact, thereby forming tumors. Thus, one assay for cancer cell growth inhibitor compounds measures the ability of cancer cells to grow and divide in the presence of the compound. There are many ways known to the skilled artisan in which this assay can be conducted, however two preferred methods are set forth below.

A. Stably Transfected Cell Assay

In this procedure, any human cancer cell line that does not express the DKR gene to be tested (either human DKR-1, 2, 3, 4, or a fragment or variant thereof) is transfected with the DKR gene under evaluation, where the DKR gene is inserted into a vector such as

pcDNA3.1 (Invitrogen, Carlsbad, CA) or other suitable mammalian expression vector. Transfection can be conducted as described herein. The transfected cancer cells are cultured to generate a stably transfected cell line. Once a stably transfected cell line has been established, the cells are added to Noble or equivalent agar (about 0.35 percent) prepared in standard mammalian cell culture medium such as RPMI. The cell/agar solution is poured on to petri plates containing solidified agar ban (about 0.5 percent agar). Colony formation is evaluated daily to determine the rate of growth of the cells, and culture medium is added to each plate as needed. Separately, the same cells are transfected with vector only (containing no DKR gene). These "control" cells are then treated in an identical manner to the DKR gene containing cells and can be used as a standard of comparison for the DKR gene containing cells.

Examples of suitable cancer cell lines for conducting this assay include, without limitation, human breast cancer cell line MCF7 and the glioblastoma cell line U-87MG.

B. Protein Assay

An alternate or additional assay to measure the growth of cancer cell lines treated with a DKR polypeptide is as follows. Any human cancer cell line not expressing the DKR polypeptide under evaluation can be cultured and prepared with an agar solution as described above. The cells can then be plated as described, and a solution of DKR polypeptide (either full length, or a fragment or variant thereof) in culture medium can be added to the agar either daily, every other day, or once per week for three weeks.

Typically, a concentration of about 10 nM will be added, although a series of dilutions ranging from 1 nM to 1 mM can be used. Control plates will receive a solution of culture medium only. The plates can be monitored daily for up to about three weeks to evaluate cell colony formation. After three weeks, control and experimental plates can be compared for the number and size of cell colonies. It is anticipated that those plates receiving DKR polypeptide that is biologically active will have fewer cell colonies, and the colonies will be smaller, as compared to control plates.

Example 13: *In Vivo* Tumor Assay

The ability of each DKR polypeptide to inhibit tumor growth *in vivo* can be evaluated as follows. Tumor cells not expressing the DKR gene under evaluation can be transfected using procedures described herein with a DKR nucleic acid construct encoding a full length DKR gene, or a fragment or variant thereof. The transfected cells can be maintained in culture (as described herein) until ready for use.

Male or female athymic nude mice (Charles River Labs, Boston, MA) are kept in a sterile environment. The mice are then injected with about 2 x 10^6 cells (either DKR transfected cells or control "vector only" transfected cells) in a total volume of about 0.1 ml can be injected sub-utaneously. The mice can then be examined daily for appearance of (a) tumor(s) and for the size of the tumor. Preferably, the mice will be examined for up to about six months so as to provide time for tumor growth (and regression where DKR polypeptides are effective at decreasing tumor growth). The tumor(s), where present, can then

be removed, weighed and examined for (1) the presence of DKR polypeptide, and (2) morphology. Tumors from mice containing DKR construct transfected cells can be compared to tumors from mice containing cells transfected with vector only. It is anticipated that DKR polypeptides, due to their similarity with *dkk-1*, a potent *wnt8* antagonist, will be able to decrease the size of the tumor as compared with controls.